THE THERMOPHILIC AEROBIC SPOREFORMING BACTERIA

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I. THE ORGANISMS

1. *Introduction.* The thermophilic aerobic sporeforming bacteria have been objects of scientific interest since the isolation of the first bacterium of this type by Miquel (118, 119) in 1879. Microorganisms living at high temperatures had been known since the early years of the nineteenth century, but work on them was limited to observations of more or less spectacular growths in hot springs. Miquel's organism, on the other hand, was isolated from the waters of the Seine; scarcely a place obviously favorable for the development of a microbe which could grow at 73 C, and incapable of growth at low temperatures.

During the following decades, the thermophilic microorganisms were the subject of a lively field of investigation, and many of the fundamental facts of their existence were discovered during this period. Thermophilic bacteria were found in almost every sample of soil, mud, or water which was examined. They were isolated not only from tropical soils (98, 99) and desert sands (128), but also from air (119, 149), freshly fallen snow (79, 142), sea water (109), and recently even from ocean bottom mud (13). Water (12, 22, 75, 122), sewage (12, 58), feces of man and various animals (4, 8, 19, 23, 27, 77, 109, 119, 168), cultivated soils (23, 37, 66, 77, 109, 166, 167), and masses of decaying plant materials (43, 55, 81, 86, 112, 114, 173, 174) were especially good sources. The great majority of the bacteria isolated at high temperatures were proved to be aerobic sporeforming bacteria; nonsporeformers were rare, and facultative forms, more common than obligate anaerobes. Although strictly anaerobic thermophilic bacteria are

known, experiments designed for their isolation often yield facultative aerobes (52, 111, 134).

Since the germs of thermophilic aerobic sporeforming bacteria are universally distributed, it is evident that proliferation of these organisms will take place whenever favorable circumstances occur, whether in nature or under artificial conditions. The temperature conditions are obviously favorable in hot springs, but the supply of organic matter in most thermal waters is low, and autotrophic thermophilic sporeformers, if they exist at all, are rare; hence the sporeforming bacteria do not play a very important role in the economy of hot springs unless masses of algae accumulate and decay.

A number of early investigators (98, 99, 149), attempting to account for the general distribution of thermophilic bacteria, found that temperatures in the upper layers of the soil were high enough to support the growth of thermophils for several hours each day, at least during the summer. Whether thermophilic bacteria develop extensively in the soil outside of the tropics, however, has remained questionable.

Mass development of thermophilic bacteria usually occurs in tightly packed masses of plant material, such as haystacks or manure piles containing considerble straw. In haystacks, for example, high temperatures are likely to develop, and, unless special precautions are taken, the hay may spontaneously ignite. Along with the rise in temperature a loss in weight has been noticed, and gaseous products and volatile acids are formed (26, 56, 112, 114). For some time there was a controversy as to whether the spontaneous combustion of hay was due to purely chemical causes or whether heating by microbial action was a predisposing factor. This discussion was settled largely by the work of Miehe (114), who isolated a number of bacteria and fungi from masses of heating hay. Among these microbes was a sporeforming bacterium so active in raising the temperature of masses of plant material that the heating observed under natural conditions could be duplicated with sterilized hay and a pure culture of this bacterium, appropriately named *Bacillus calfactor*. Since then, alteration of plant material under the action of thermophilic bacteria has been deliberately used in the production of certain types of fodder and manure (57, 148, 159), while the conditions favoring the development of thermophilic bacteria in hay are well enough known to avoid their extensive growth when it is not desired.

In general, however, thermophilic bacteria are a nuisance rather than a help in agricultural and industrial practice. Aerobic, facultatively anaerobic, and anaerobic thermophilic sporeformers are among the principal causes of spoilage of canned foods since not only are their vegetative cells adapted to life at elevated temperatures, but also their spores are unusually heat resistant, some surviving even half an hour of autoclaving (32, 33, 42, 125, 152, 169). The thermophilic bacteria are especially troublesome when canned products are stored at temperatures above 30–35 C, as in the tropics (14), since contamination with viable spores of thermophils cannot be easily detected until the food is exposed to high temperatures.

Sometimes the bacterial count of milk increases during pasteurization, due to

development of thermophils during the heating process (59, 89, 104, 147, 152). Canned milk is also subject to occasional attacks of coagulation by thermophilic bacteria (87, 147). Opinion has differed on the importance of these microbes in dairying, some tending to dismiss them since they are nonpathogenic and do not develop extensively in milk stored at low temperatures, others considering them as soil microorganisms, indicators of general contamination of milk (10, 59, 84a, 160).

Thermophilic sporeformers are often a problem in sugar refineries, where they have been observed living and fermenting in syrups containing up to 40% sucrose (29, 30, 31, 33, 42, 101, 102). Other thermophils or near-thermophils cause a ropy spoilage of bread (85). Some ingredients of culture media, such as the agar which was produced in this country during the war years, are at times contaminated with thermophilic bacteria, which are extremely troublesome in bacteriological work (124, 125).

Although a good deal of information on ecology and some scattered bits of knowledge of the physiology of the thermophilic bacteria were gained by the early investigators, a large part of the extensive literature on thermophils is concerned with documentation of their occurrence in various spots, or with descriptions, often not related to the findings of others, of organisms isolated. Relatively little knowledge of the general biology, physiology, and biochemistry of the thermophils has been collected from the time of the first flurry of interest until recently. Crude cultures were early shown to be able to decompose cellulose, denitrify, and fix nitrogen (90, 97, 98, 99, 120, 132, 138, 139, 140), but the bacteria responsible for these processes were not isolated and studied; cellulose decomposition and N_2 fixation in particular require more investigation. The principal biochemical activities studied with pure cultures are the digestion of starch and proteins (12, 27, 44, 45, 96, 98, 99, 109, 113, 133, 149, 152, 153), and the fermentation of sugars to yield acidic products (44, 45, 85, 96, 101, 152). Little is known concerning the types of substances that can be decomposed by specific thermophils and the nature of the decomposition products, in spite of two ambitious attempts to study these problems (12, 96). Until very recently, the systematics of the thermophilic sporeformers, like that of their mesophilic counterparts, left much to be desired. However, now that the work of Gibson (76), of Smith *et al.* (155), and of Knight and Proom (93) has done much to clarify the status of the mesophilic bacilli, a more satisfactory ordering of the thermophilic forms has likewise become possible.

The most interesting problem posed by the thermophilic microorganisms is a physiological one. How are these microbes able to live and grow at temperatures so high that many proteins are coagulated and the existence of life appears as a biochemical anomaly? Some speculations have appeared concerning this problem, but few experiments have been conducted. It was shown early that thermostable hydrolytic enzymes could be isolated from culture filtrates of thermophilic bacteria (133, 139, 140), and recent work has extended this finding of thermostability to other types of proteins within the cell (74, 108, 115, 116, 117).

It is by no means certain, however, that this can in itself account for the ability

of these organisms to grow at high temperatures, and no publications exist in which an attempt is made to assess the different factors which may contribute to a thermophilic habit of life. Other mechanisms, ranging from an insulating shell (64) to a great capacity for regenerating proteins destroyed by heat (2), have been proposed; a critical resumé of the various possibilities appears desirable. Chemical investigation of the difference between thermostable and thermolabile proteins is a problem for the future.

Since the thermophilic bacteria presented so many unsolved problems, and since the mechanisms permitting growth at high temperatures seemed of particular biochemical importance, an investigation of such microorganisms was begun in this laboratory some five years ago. The thermophilic aerobic spore-forming bacteria were selected as the principal objects of attention since they are the most common and accessible organisms capable of growth at elevated temperatures.

It soon became evident that more information concerning the bacteria themselves, their habits, and their relations to other forms was a desirable prerequisite to a deeper understanding of their metabolism. The literature on thermophilic bacteria indicates that interest in these microorganisms, especially in their more fundamental scientific aspects, has been oddly sporadic, with the result that much the same experiments have been done over and over again. Aside from passing mention, earlier work appears to have been neglected rather than assimilated and used as a base for the gathering of new knowledge. An integrated treatment of the field thus appeared highly desirable.

The term "thermophilic bacteria" has meant many things to many writers. Some have called a bacterium capable of growth at 50 C a thermophil, while others have restricted the name to microbes with a minimum temperature of growth of 45–50 C, designating those with a lower minimum and a maximum temperature over 50 C as "thermotolerant" (149, 153). Recent usage, which will be followed in the present discussion, has tended to refer to any microbe capable of growth at 55 C as a thermophil (71, 80).

For two reasons, most attempts at a more detailed nomenclatural separation of bacteria into groups based on temperature ranges for growth appear today to have only limited value. First, there is a continuous series of maximum and minimum temperatures for growth; at no point can there be found a temperature such that microbes growing above this point have other properties distinctly different from those growing below. Second, it has been found that different isolates of a single microorganism may possess different temperature requirements for development, and that the temperature range of an isolated culture may be varied by suitable experimental procedures. Some terms to describe the growth habits of microbes with respect to temperature are, however, useful. For differentiating between microorganisms capable of growth at both high and low temperatures and those which are restricted to elevated temperatures for their development, there appears to be little difference whether the older terminology of facultative and obligate thermophil (19, 20, 32, 114, 123) or the newer one of eurithermal and stenothermal organism (88) is used.

2. *Isolation and characterization of pure cultures of thermophilic bacteria.* It is obviously desirable that the members of a group of microorganisms be described and classified in a way which will make them readily recognizable. For the thermophilic aerobic sporeforming bacteria, this has been a difficult task. Most workers who have in the past tried to characterize thermophilic sporeformers can be divided into those who have named almost every isolate as a new "species" and those who have considered all of them to be much alike. The second view has generally been favored by investigators who have studied the group extensively enough to be aware of the variability of its members (23, 27, 80, 122). Especially since the studies of Smith and his co-workers (80, 155) it is clear that the range of variation among sporeformers in general is sufficiently large that the most useful taxonomic distinctions are those drawn with a broad brush.

A survey of the literature suggests, however, that the monotonous picture presented (at least to the "lumpers") by the thermophilic bacilli might reflect the limited range of conditions of isolation used by their investigators. Most of the cultures described have been isolated by incubation of samples of soil or other inocula in complex nutrient media, or by direct plating from samples of food products, plant material, or soil onto peptone, meat extract, yeast extract, and similar agar media. It is evident that the first procedure is an elective culture, while the second leads to the isolation of only those bacteria which are most abundant in the soil or food products. Since many foods, in particular, may be considered as natural enrichment media not very different from the complex nutrient media of the laboratory, it is perhaps not surprising that these procedures have usually led to the isolation of much the same kind of thermophilic bacteria.

On the other hand, a relatively large number of biochemical processes has been observed with crude or undescribed cultures of thermophils, so that more types of bacteria might be expected to be obtained by a systematic, deliberate use of elective culture techniques. While a number of investigators have used enrichment cultures to obtain thermophils capable of decomposing specific carbohydrates, the only general survey of the thermophilic microflora using this technique is that of de Kruijff (Kruyff) (98, 99), and the extensive account of the bacteria so isolated, which he promised, never appeared. He obtained proteolytic, amylolytic, and lipolytic bacteria, denitrifiers, nitrogen fixing bacteria, and some which decomposed cellulose from enrichment cultures inoculated with tropical soils, but did not describe the isolates in detail.

Further experimentation along this line appeared worth while; accordingly, a wide variety of enrichment culture media, as shown in Table 1, was used to obtain cultures of thermophilic bacteria. The cultures were inoculated with soil, freshwater or marine mud, or dry plant material. Cultures were incubated aerobically at 55 or 65 C.

Thermophilic bacteria developed more or less abundantly in all cultures except those containing formate, the amines, *tert*-butanol, ethylene glycol, phenol, indole, thioglycolate, barbiturate, and paraffin. In some of the others, notably the pectin and chitin enrichments, growth was light and there was little

TABLE 1

Media used for enrichment cultures

A. *Complex media.*

10% yeast autolysate
10% yeast autolysate, 2% glucose
10% yeast autolysate, 2% glucose, 2% $CaCO_3$
10% yeast autolysate, 5% urea
1% peptone

B. *Simple media*, containing as mineral base 0.1% ammonium sulfate, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, in tapwater, with the following C sources:

sugars (0.5–1%)	*monohydric alcohols* (0.1%)	*polyhydric alcohols* (0.5–1%)
glucose	n-capryl alcohol	ethylene glycol
fructose	*tert*-butanol	glycerol
galactose	cyclohexanol	sorbitol
sucrose		mannitol
maltose		inositol
raffinose		
arabinose		

carboxylic acids (as Na salts, 0.1%)	*amino acids* (0.2–0.5%)	*amines* (as hydrochlorides, 0.1%)
formic	casein hydrolyzate	ethylamine
acetic	asparagine	triethylamine
propionic	Na glutamate	creatine
caprylic	glycine	
oleic	alanine	
lactic	histidine	
succinic	tyrosine	
malic	tryptophan	
citric	cystine	
tartaric		
glutaric		
oxalic		
thioglycolic		

aromatic compounds (0.1%)	*heterocyclics* (0.1%)	*hydrocarbons*
phenol	indole	paraffin
Na benzoate	nicotinic acid (Na salt)	
Na p-hydroxybenzoate	Na barbiturate	
Na salicylate	uracil	
	adenine	

natural high polymers

cellulose
agar
starch
inulin
pectin
chitin

evidence of decomposition of the polymer. Whether or not the inoculum was pasteurized, only sporeforming bacteria developed in the enrichment cultures; never did the type of inoculum influence the results obtained.

These cultures, while covering a much wider range of conditions than those used by earlier workers, still leave many possible environments for growth of

thermophilic bacteria unexplored. Moreover, growth in such a culture does not necessarily imply that the bacteria isolated from it are capable of using the substrate supplied, nor that materials unattacked by thermophils in media such as these may not be subject to microbial attack at high temperatures. Many thermophilic sporeforming bacteria require organic growth factors for their development; the enrichment media containing a single carbon source in a mineral medium tend to select against such organisms, even though a certain quantity of growth factors is supplied by soil in the first crude cultures.

The growth factor requirements of all the thermophils which have been encountered in this study are met readily by the use of a complex medium such as yeast extract; hence the following isolation procedure was adopted. Transfers were made from the elective cultures to fresh media of the same composition, and from these pure cultures were isolated by two successive platings on yeast autolysate agar at 55 or 65 C. A few strains from cultures containing sugars were isolated on yeast agar with glucose, or with glucose and calcium carbonate. Some isolations on minerals plus single carbon source media were tried; these led to progressively weaker growth, which could be restored to vigor by the addition of yeast autolysate to the medium. Attempts to substitute for yeast extract mixtures of vitamins and other growth factors did not lead to satisfactory results for reasons which will be discussed when the nutritional requirements of the thermophilic sporeformers are considered.

With favorable media, the entire process of isolation, including obtaining growth in an enrichment culture, transfer to a fresh medium of the same composition, and two successive platings on agar medium could be completed in 48 hours or less. Use of these short time intervals minimizes the difficulties due to drying out of media which have plagued many earlier workers. Cultures were maintained on yeast autolysate agar slants. Transfers were made at intervals of one to two months, the freshly inoculated slants being incubated at 55 C until good growth and sporulation were obtained (usually 24 hours), then stored at room temperature.

The methods used for characterization of cultures were in general those developed by Smith and his co-workers (80, 155) with a few modifications which appeared desirable for the thermophilic sporeformers. Microscopic and macroscopic examination of isolates, tests for hydrolysis of gelatin, starch, and casein, and determination of maximum temperatures of growth were performed as described. Since it has been pointed out by Gordon and Smith (80) that temperatures of media in incubators may differ greatly from the putative temperature of the incubator, all experiments in which temperature control was important were carried out in a thermostatically controlled water bath.

Nitrite formation from nitrate was tested by the Griess-Ilosvay method; production of gas in nitrate broth was also noted when it occurred. Because of the growth factor requirements of many of the thermophils, the media described by Smith *et al.* for determination of utilization of nitrate as a source of nitrogen and of citrate as a source of carbon did not appear to give a fair measure of the property intended to be tested. To circumvent this difficulty, a small amount of yeast

autolysate (*ca.* 0.01%) was added to the citrate and glucose-nitrate media of Smith *et al.* Control tubes containing the same medium with the citrate or nitrate omitted were also inoculated and growth and pH changes in the two sets of cultures compared. It was found possible in this way to obtain clear-cut tests for citrate and nitrate utilization.

Tests for growth at an acid reaction in glucose nutrient broth yielded erratic results. Measurement of the pH range for growth was therefore made in buffered media containing M/15 potassium phosphate mixtures, 0.1% ammonium sulfate, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% glucose, and 0.02% Difco yeast extract. All cultures isolated grew in this medium at pH 7.

The ability of strains to develop anaerobically in glucose media was noted. 55 C was selected as the standard temperature for carrying out determinative tests on the isolates. Several cultures which were able to grow well at lower temperatures were also tested at 30 C. No significant differences were noticed between the sets of determinative characteristics observed at the two temperatures.

The rapidity of growth and senescence in cultures at elevated temperatures made it desirable to use shorter time intervals for incubation than those recommended for the mesophilic sporeforming bacteria. For gram staining and observation of motility, vegetative cells not over 8 to 10 hours old were used with satisfactory results. Biochemical tests were usually completed in one to two days; further incubation did not lead to any increase in positive results.

The procedures for isolation, cultivation, and testing have been described in some detail since the experience gained in this study indicates that the thermophilic aerobic sporeforming bacteria are protean creatures, and that the characteristics of laboratory strains depend to a considerable extent on the conditions used in handling them. Similar observations have been made by earlier workers; in 1902 Blau (25), who provided some of the first careful descriptions of thermophilic bacteria, noted that their characteristics tended to change on continued culture in the laboratory; Bruini (27) wrote of some thermophilic bacteria in 1905, "Einige Arten, die ich zuerst vom Standpunkte der Morphologie und der Züchtungskennzeichen aus für verschieden hielt, haben zuletzt vollständig dieselbe Form angenommen". Winogradsky's dictum (178) that the pure cultures of the laboratory are like plants maintained for a long time in botanical gardens applies with particular force to this group of bacteria.

Fresh isolates tend to display a bewildering diversity of characteristics, while strains which have been maintained in culture for some time are usually readily classifiable. Some examples of changes in characteristics during culture under laboratory conditions are given in table 2. One hundred and five isolates have been studied. Most of these, after adjustment to artificial culture conditions, could be placed in one of four groups:

Group 1 contains bacteria whose properties are reminiscent of *Bacillus circulans*. Vegetative rods measure 3–6 by 0.8–1.0 μ. Spores are oval, terminal, and swell the sporangium. A thin, translucent, spreading growth on agar is generally characteristic; some strains form motile colonies which move over the agar surface. The minimum temperature for

growth is 35 C; the maximum 65–70 C depending upon the strain. Growth occurs at pH 6 and higher, but not at pH 5. Growth on glucose nutrient agar is the same as, or less than, that on nutrient agar without glucose. Starch, gelatin, and casein are hydrolyzed by most strains. Nitrate is not used as nitrogen source; some strains reduce nitrate to nitrite, others do not. Acetoin (acetylmethyl carbinol) is not produced by any of the isolates studied here. The majority of the strains of this group examined in the present study are strict aerobes; however, four isolates were obtained from contaminated agar which can develop anaero bically in glucose media. These isolates also differ from the other cultures in that they can utilize citrate and hydrolyze agar. This last property tends to weaken on continued culture.

In general, the cultures of this group resemble *Bacillus stearothermophilus* Donk, as extended by Gordon and Smith. Comparison with an authentic strain of *B. stearothermophilus* (N. R. Smith's no. 28) confirmed this conclusion.

Representatives of group 1 may regularly be obtained from enrichment cultures in media containing amino acids as source of carbon (and nitrogen, if desired), incubated at 55–65 C. It was found to be the most abundant group in a number of soil samples which were tested, so that representatives may also be isolated from soil by direct plating.

TABLE 2

*Effect of laboratory culture on certain characteristics of thermophilic aerobic sporeforming bacteria**

	FRESHLY ISOLATED	AFTER TRANSFERS
	strains	*strains*
Growth at 30 C	4	8
Growth at 35 C	13	21
Acetoin production	0†	18
Hydrolysis of gelatin	11	16
Hydrolysis of starch	15	20
Spores oval, terminal, swelling sporangium	5	4
Spores central, swelling sporangium	4	1
Spores terminal, not swelling sporangium	12	16

* Comparison of properties of 21 strains when freshly isolated and after 4 transfers at 55 C, separated by 4–5 days storage at room temperature.

† Many cultures grew poorly.

Group 2 develops preferentially in carbohydrate media which are not buffered, so that they become acid as bacteria develop, or in similar media which have been initially slightly acidified (to pH 5–6) with lactic acid and incubated at an elevated temperature. Knight and Proom (93) obtained them using milk as culture medium and incubating several days at 45 C. The isolates so obtained could all be identified as more or less typical strains of *Bacillus coagulans* Hammer, emend. Gordon and Smith. Vegetative rods measure 2–5 by 0.7–0.8 μ. Spores are oval, terminal to subterminal, slightly swelling the sporangium. Growth on agar is usually smooth, white, not as spreading as that of most cultures of Group 1. The minimum temperature for growth is *ca.* 35 C, the maximum 60–65 C. All strains grew at pH 5 and usually more copiously in the presence of glucose. Acetoin production, considered to be a characteristic of *B. coagulans* (80), was observed with most strains but was occasionally weak or lacking. Starch is hydrolyzed; gelatin and casein are not. A few isolates give a weak nitrite reaction when cultured in nitrate broth. Nitrate is not utilized as nitrogen source. Citrate is not utilized. The bacteria can develop anaerobically in glucose media. Glucose broth is made strongly acid (pH 4-5).

Group 3, which in general resembles *Bacillus subtilis*, is the most biochemically versatile, and the most variable, of those encountered. The phenomena of adaptation to laboratory

conditions appear most strikingly in this group, as has long been known. Schardinger (152) wrote of them in 1903,

> "Die durch fortgesetzte Kultur auf den gebräuchlichen Nährboden gefördete Variationsbildung scheint mir innerhalb dieser Gruppe eine bedeutende zu sein, so dass es wenig Gewinn bringen möchte, in geringem Grade abweichende Arten gesondert anzuführen. Ihrem ganzen Verhalten nach gehören die zu schildernden Keime der grossen Gruppe der Heu-Kartoffel-Bacillen an, für die ja schon Flügge eine grosse Akkomodationsbreite hinsichtlich der Temperatur feststellte."

Bacteria of this type may be obtained from enrichment cultures in carbohydrate media which are buffered with calcium carbonate so that they do not become acid. Such sporeformers also come to the fore in many of the more "unusual" enrichment media; *e.g.*, those containing aromatic compounds, heterocyclic compounds, alcohols, etc.

The vegetative rods are usually 3–6 by 0.7–1.0 μ. Spores are characteristically oval to cylindrical, terminal, and do not swell the sporangium; but some isolates have been obtained in which the spores are oval, central, and do swell the sporangium. On culture for some months these forms give way to the typical cylindrical sporangia. Sames (149) earlier noted this type of variation. Growth may be singly, in pairs, or in short chains. Colony types are highly variable; one which is frequently observed has a smooth center, with a dull matte finish, and a finely fimbriate edge. Smooth, glossy, nonspreading colonies are also common.

The temperature range for growth depends upon the temperature of isolation. Strains isolated at 55 C grow from 55–60 C down to room temperature, while isolates obtained at 65 C often have a minimum of 40–45 C and a maximum of 65–70 C. After some transfers grown at 55 C and stored at room temperature, strains which originally had high minimum temperatures gradually acquire the ability to grow at room temperature.

Some strains of this group are facultative anaerobes, others, strict aerobes. Acetoin is produced. Starch, gelatin, and casein are hydrolyzed. Nitrate is reduced to nitrite, and some cultures are vigorous producers of nitrogen gas. Nitrate can be used as nitrogen source for growth. Citrate is utilized. Growth occurs at pH 6 and above, but not at pH 5; the limiting pH in glucose broth is 5.5–6; many strains make the broth alkaline.

Group 4 comprises bacteria with a round terminal or subterminal spore which swells the sporangium. These may be obtained by enrichment in media containing salts of carboxylic acids as well as in the yeast extract–urea medium. None of the isolates obtained required urea or ammonium carbonate for growth. Two strains formed motile colonies on agar plates.

Bacteria of this group develop between 35 and 65–70 C. Nitrate is not reduced to nitrite, nor is it used as nitrogen source for growth; some strains utilize citrate. Gelatin and casein are hydrolyzed, but starch is not. Acetoin is not produced. Growth is best in slightly alkaline media.

These characteristics, with the exception of temperature range, are closely similar to those of *Bacillus sphaericus*.

3. *Relation of previously described thermophilic sporeformers to these groups.* Recent writers on the subject of thermophilic bacteria have tended to dismiss the descriptions of early workers as being too fragmentary to be useful. While many early accounts deserve this neglect, others, those of Sames (149), Schardinger (152), Blau (25), and Miehe (114), for example, present readily recognizable pictures of their organisms. In addition, many thermophils have been characterized in a way which permits their probable assignment to one of the groups encountered in the present study since these groups are defined by morphology and by a general pattern of physiological characteristics, rather than by small details which are, as has been mentioned, too variable in this group to be of use.

From the published descriptions (32, 42, 54) and from the recent careful work of Smith *et al.* (80, 115), it may be concluded that most of the aerobic or facultatively anaerobic thermophilic bacteria found as contaminants in canned foods belong to groups 1 and 2, *B. stearothermophilus* and *B. coagulans*. It is not always possible to tell from published work which of these types was encountered; both develop acid but no gas in sugar containing media; they are morphologically quite similar, and differ principally in that *B. stearothermophilus* has a somewhat higher temperature range for growth, while *B. coagulans* develops in more acidic environments and lacks the proteinases and nitratase possessed by most sporeforming bacteria. In Cameron and Esty's (32) classification of bacteria found in spoiled canned foods, their Group 80 suggests *B. coagulans* (except for their mention of polar flagella, which does not fit any known *Bacillus*), while their obligate thermophils of Group 100 are undoubtedly *B. stearothermophilus*. McClung (111) states that *B. stearothermophilus* is the type species of the bacteria responsible for "flat sour" spoilage. It is evident that strains of *B. stearothermophilus* which develop extensively in canned foods must be facultatively anaerobic although sufficient air may be left in cans to permit growth of 100 to 500 thermophilic bacteria per ml, with consequent slow spoilage of the food (129).

Miehe's *B. calfactor*, which he isolated from hay undergoing spontaneous heating, can be placed with some assurance in Group 1 since it was described as a strictly aerobic bacterium, developing only at high temperatures, bearing a terminal oval spore which swells the sporangium, and forming motile colonies on agar plates. Most other early workers had among their cultures some which appear to belong to this group, if one admits a good deal of variation in proteolytic and amylolytic properties. These characteristics are known to vary from strain to strain, and many workers may have prepared their descriptions before their cultures became stabilized under laboratory conditions, hence admitting such variation seems permissible. In general, it may be concluded that bacteria of Group 1 are the thermophils which have been most commonly reported.

Thermophilic bacteria similar to *B. subtilis* are almost as common, however, and from the early literature on there appear a number of accounts of such organisms. Schardinger's cultures (152) have already been mentioned; Sames (149) before him isolated thermophils which clearly fall in this group; Tsiklinsky (167) considered one of her isolates to be identical with *B. subtilis* except for its temperature range; Laxa (102) obtained bacteria at 73 C which "dem *Bacillus subtilis* ähnlich war"; Blau (25) also recognized such similarity. Coolhaas' (44, 45) *Bacillus thermoamylolyticus* was clearly a *B. subtilis* type; Adant (1) mentions similar forms; and Verhoeven (170) recently isolated from spoiled cured ham thermophilic bacteria whose properties other than temperature range were identical with *B. subtilis* as defined in the monograph of Smith *et al.* (155). Since, as will be seen, many properties of thermophilic *B. subtilis* strains are distinctly different from those of the *B. circulans* group (Group 1), much confusion has been engendered by attempts to repeat work done on organisms of one type with thermophils of the other. The greater accommodation of thermophils of the *B. subtilis* group to artificial culture media, including their tendency to lose an

obligately thermophilic character on continued cultivation, has already been mentioned; it will be shown later that these two groups also differ markedly in the properties of certain of their enzymes.

Thermophilic bacteria of the round-spored type, Group 4, do not appear to have been encountered by earlier workers. Almost the only organism which might fall in this group is one described by Catterina (36), and not enough information was given about the bacterium to make possible a decision on this point. There is also a passing mention by de Kruijff (98, 99) of a round-spored thermophil.

In addition, there exist a few descriptions of thermophilic aerobic sporeforming bacteria which appear significantly different from those in the four classes discussed here.

1. Ambrož's (4) *Denitrobacterium thermophilum:* Rods 3.5–7 by 1–1.8 μ; terminal oval spores, slightly swelling the sporangium; colonies on agar resembling those of *Bacillus mycoides*, nitrate and nitrite converted to N_2, active denitrification occurring even in 3% $NaNO_2$(!); no hydrolysis of gelatin nor of starch; no growth on glucose or glycerin agar, nor in milk; temperature range for growth 37 to 65–70 C.

2. Coolhaas' (44, 46) *Bacillus thermocellulolyticus:* The only definitely aerobic thermophilic cellulose decomposer isolated before the work of Murray (126), unless one credits the casual remark of Krohn (96) that one of his strains (isolated on meat extract) could utilize this carbohydrate. Rods 3.5–4 by 0.3 μ; spores oval, excentric, swelling the sporangium. Colonies on cellulose agar show a zone of clearing, due to hydrolysis of cellulose, around the periphery of each colony, but cellulose is not decomposed in liquid media, neither aerobically nor anaerobically. No acid nor gas from carbohydrates other than cellulose and starch. Very weak growth in meat extract or glucose media. No nitrate reduction nor proteolytic action. Temperature range 35–65 C.

3. Campbell's (34) *Nitrosobacillus thermophilus:* The only thermophilic nitrifier mentioned in the literature. de Kruijff, the only other investigator who has reported attempts at isolating such thermophilic bacteria, had no success. Rods 3.8–8 by 1–2 μ, single and in chains. Spores terminal, swelling sporangium. Ammonia oxidized to nitrite, most actively at pH 9.5. Growth also occurred on all ordinary organic media; CO_2 was necessary for growth.

The work on these apparently exceptional thermophils is lacking in completeness and, possibly, in a critical attitude toward experimental data. More work on such organisms would be desirable since they represent the only notable exceptions to the generalization that all thermophilic aerobic sporeforming bacteria so far described fall within a few broad but reasonably well defined groups.

4. *Thermophilic variants of mesophilic sporeformers.*

Es ist nicht ausgeschlossen, dass es einige wenige Organismen gibt, die wirklich an Standorten mit hoher Temperatur (heisse Quellen, gärende Düngerhaufen, usw.) vollkommen angepasst sind und als abgeschlossene, selbstständige Formen (Arten) anzusehen sind. Die grosse Masse der in der Natur überall verbreiteten thermophilen Organismen sind aber sicher nur durch die künstliche Kultur bei hohen Temperaturen entstandene Mutationen von Formen, die sich auch bei gewöhnlichen Temperaturen normal entwickeln.—Lieske.

The majority of those who have experimentally investigated the problem of thermophily have based their studies on the assumption, expressed or tacitly im-

plied by the direction of their work, that the thermophilic microorganisms are more or less closely related to present day microbes growing at ordinary temperatures. Other hypotheses have played little part in experimental work although they have often figured in general discussions. Mention may be made of the idea, advanced by Weed (177) and by Ambrož (5), that thermophilic microbes are survivors of a past geological age, and of Arrhenius' (9) concept that they are visitors from another planet.

The views held as to the nature and closeness of the relation between thermophils and mesophils have, however, varied. In the early years of this century a very close relationship was generally accepted. Rabinowitsch (142), Schillinger (153), Tsiklinsky (167, 168), and Miehe (114) all considered thermophilic bacteria to be variants of mesophilic forms. Tsiklinsky, starting with a mesophilic *B. subtilis* strain, had derived a thermophilic variant from it which closely resembled the parent culture except for its temperature range.

Later, however, these findings and this attitude came to be regarded with skepticism. Although Jancke (89) claimed to have obtained thermophils from ordinary strains of *Bacillus mesentericus* and also to have obtained *B. mesentericus* from thermophilic cultures, his experiments were not conclusive. Attempts by Golikowa (79) and by Casman and Rettger (35) to acclimatize sporeforming bacteria to temperature ranges different from those in which they normally occurred met with at best very limited success. These workers tried to effect the conversion by a process of gradual adaptation. Tsiklinsky (167, 168) gave few experimental details concerning the transformation of *B. subtilis;* Dallinger's (50, 51) experiments, in which protozoa were trained to grow at 70 C by gradual adaptation, were well known; and general acceptance of the concept that heritable changes in microorganisms proceed through mutation and selection rather than by small adjustments to the environment was still in the future. Even as late as 1945, Imšenecki and Solnzeva (88) considered that thermophilic bacteria were probably derived from mesophilic forms by gradual adaptation, in spite of the negative outcome of experiments in this direction.

But at the same time that this skeptical attitude toward thermophilic variants was prevalent, arguments were being developed which made the derivation of thermophilic microorganisms from mesophilic parents almost a logical necessity. These arguments arose from the ubiquitous occurrence of obligately thermophilic microorganisms in environments in which their development seems impossible. This problem had early been recognized, and various solutions had been proposed. Although it had been shown (77, 96, 98, 99, 120, 130, 149) that the soil, when warmed by the summer sun, reaches a temperature high enough to permit growth of thermophilic bacteria, doubt remained whether outside of the tropics these temperatures were maintained long enough to permit the bacteria to conclude a cycle of growth and sporulation. Koch and Hoffmann (94) suggested that thermophils may grow at lower temperatures in the soil than in the laboratory.

Distribution of thermophilic bacteria from natural sources permanently or temporarily maintained at high temperatures had also been considered (27, 43,

98, 99, 114, 168). Hot springs are an obvious example of such a source; haystacks and manure piles and the intestines of warm-blooded animals are other places where thermophilic bacteria might develop and from which they could be spread throughout the soil. But in this case a correlation should exist between the numbers of thermophilic bacteria and the closeness of association of the soil sample chosen with such sources of heat. None was found; thermophilic bacteria have have been isolated from soil deep in caves (105) and, more recently, from ocean bottom mud (13), where contamination from hot springs, hay, or warm-blooded animal excreta seems most unlikely.

These arguments, developed by Lieske (105) in 1926, led him to the conclusion that the most likely explanation of the ubiquitous occurrence of thermophilic microorganisms was their selection, as variant forms of the natural mesophilic population, or as mutants induced by the high temperature, whenever the requisite thermal conditions were supplied. Lieske's experimental evidence for this view was, however, slight. His studies were carried out with thermophilic actinomycetes. The spores of these microbes, unlike those of the sporeforming bacteria, are killed by prolonged exposure to cold. Samples of soil were held at low temperatures for periods of time sufficiently long presumably to destroy the spores of any preexisting thermophilic actinomycetes, and strains capable of growth at high temperatures were isolated from these soils, but attempts at isolation of thermophilic variants from pure cultures of mesophilic actinomycetes were unsuccessful. It was, however, possible to obtain mesophilic strains of actinomycetes from thermophilic ones.

Stronger evidence of variation from mesophily to thermophily was provided by the experiments of Kluyver and Baars (91) with *Desulfovibrio desulfuricans*. With this anaerobe it was possible to change mesophilic strains into thermophilic ones, and *vice versa*, by a series of transfers at increasing or decreasing temperatures, respectively. It is not entirely clear from the description of their experiments whether this result should be ascribed to adaptation or to selection. The generally negative outcome of experiments in which gradual adaptation of bacteria to growth at high temperatures has been attempted, together with the fact that large inocula were used in transfer, makes selection likely.

With this background and with the close similarity in properties which had been observed between mesophilic and thermophilic aerobic sporeforming bacteria, experiments on the selection of thermophilic variants of mesophilic sporeforming bacteria appeared worthwhile. These experiments have shown clearly that thermophilic bacteria can be derived experimentally from mesophilic members of the genus *Bacillus*.

The procedure which has generally been followed in the isolation of thermophilic variants is as follows: A population of the mesophilic sporeformer under investigation is grown in a liquid medium (usually yeast extract) at a temperature favorable for its growth and sporulation (usually 35–40 C). When the culture is well grown, some fresh medium is added and the entire culture is transferred to an incubator or water bath at 55 C. After incubation for several hours at this temperature, transfers are made to fresh medium and incubated at the high

temperature. Pure cultures of the variant strains are isolated by plating from the transfers in which bacteria develop. Blanks are always included to check on the absence of thermophilic contaminants in the media, and the mesophilic cultures used are tested for their inability to grow at 55 C from the usual loop inocula.

If the bacteria grown at the lower temperature are mixed with agar before placing at 55 C, less consistent results have been obtained, but the process of development of thermophilic strains can be followed in more detail, and some estimate of the number of individuals which can grow at 55 C can be made. The few colonies which develop in the first agar plates are composed of cells which are filamentous or otherwise abnormal in appearance, while transfers from these colonies yield normal bacteria.

Cultures of all the species of mesophilic aerobic sporeforming bacteria described in the monograph of Smith, Gordon, and Clark (155) have been tested for their ability to yield thermophilic variants. The most consistently positive results have been obtained with strains of *B. subtilis*, *B. cereus*, *B. megaterium*, and *B. circulans*. One culture of *B. pumilus* was tested and yielded a variant. *B. macerans* has not produced thermophilic strains; cultures started in yeast-glucose-$CaCO_3$ at 40 C continued to produce gas at 55 C, but transfers at this temperature failed to grow. In general, it may be stated that the strains which have given good results grow rapidly and sporulate readily. No marked qualitative effect of age of culture has been noted. Thermophilic variants can be isolated from spore suspensions as well as from growing cultures of vegetative cells.

Until such time as a mutagenic action of heat is clearly demonstrated, it appears preferable to consider the outcome of the experiments as the selection of spontaneously occurring mutants, rather than the production of resistant individuals by the action of heat. The frequency of occurrence of thermophilic variants has been determined; this evidently depends both upon the rate of production of potential thermophils and upon their success at competing with the parent type during growth at low temperatures. With a strain of *B. circulans* (ATTC 7046), where the parent has a maximum temperature for growth of 46 C, but was commonly maintained at 35–40 C, while its thermophilic variants all had a temperature minimum of 35–40 C, a consistent frequency of one thermophil in 10^6 cells of the original population was found. This is the highest frequency of occurrence of thermophilic variants observed. All the variants isolated from this *B. circulans* strain were found to be indistinguishable.

While isolation of thermophilic variants has been consistently successful from several strains of *B. subtilis* (Ford strain S8), *B. cereus* (HMS No. R.A.4.2), and *B. megaterium* (HMS No. R.A.2.2, R.A.2.3, R.A.2.4), the variants which are obtained with these are capable of growth at 30–35 C as well as at 55–60 C. Hence the variants will be in competition with the parent population, and the figures obtained for frequency of occurrence of thermophils in the population will depend on the conditions during the experiments and on the treatment of the cultures prior to the test. Figures ranging from one in 10^7 to one in 10^{10}–10^{11} have been obtained. The highest frequencies occurred when old cultures of the mesophil were transferred to liquid medium, incubated at 40 C, and then transferred to

55 C. Mesophilic cultures which had been rapidly transferred prior to the selection of variants contained fewer thermophils. Both this result and the lower frequency of occurrence of thermophils in the *B. subtilis* group suggest that the thermophilic *B. subtilis* cells are at a disadvantage in competition with the parent type at 35 C, even though the variants grow well at that temperature.

The reverse transformation, that of a thermophil into a mesophil, was noticed once. A liquid culture of a *B. circulans* type thermophil, with a minimum temperature for growth of 35 C, was allowed to stand at 30 C for three months. At the end of this time, growth of a typical mesophilic *B. cicrulans* was observed in the culture. No extensive experiments in this direction have been carried out; the results shown in table 2 indicate a tendency for variants preferring lower temperatures to develop in cultures of thermophils.

The thermophilic strains derived from cultures of *B. subtilis*, *B. cereus*, and *B. megaterium* were all closely similar and indistinguishable from isolates of Group 3, the "subtilis-type" thermophils, obtained from natural sources. No large-celled thermophilic forms were observed. The transition from a broad-celled mesophilic *B. megaterium* to a slender-celled thermophil could be clearly observed in colonies of variants developing on agar plates.

Bacteria corresponding to *B. coagulans* but with a lower or higher temperature range are not known, nor has it been possible to obtain thermophilic bacteria resembling *B. sphaericus* by selection of variants from mesophilic *B. sphaericus* strains. The similarity in properties other than temperature range between the round-spored thermophils and *B. sphaericus* is, however, so close that, considering the results obtained with other types, it appears justifiable to consider such thermophils as variants of *B. sphaericus*, even though the experimental conditions for obtaining such variants in the laboratory are not known.

A number of attempts to isolate thermophilic variants from microorganisms other than sporeforming bacteria met with, at most, limited success. Negative results were obtained with strains of *Pseudomonas*, *Escherichia*, *Aerobacter*, *Lactobacillus*, *Streptococcus*, *Mycobacterium*, and actinomycetes. In view of the established fact (105, 158a), that recent isolates of actinomycetes, in contrast to laboratory stock cultures, may display a pronounced tendency to throw off variants, experiments with representatives of this group were carried out with some 20 freshly isolated strains. The failure to obtain thermophilic offspring corroborates Lieske's earlier results; also his successful transformation of a thermophilic into a mesophilic actinomycete could be reproduced. From two freshly isolated yeasts (*Torulopsis* sp?) variants were obtained which would grow at 50 C, five degrees above their original maximum growth temperature, but these readily reverted to their original temperature range on storage at room temperature.

Since it has been shown that thermophilic sporeforming bacteria may be derived in the laboratory from pure cultures of mesophilic strains, the question may be asked whether all thermophilic sporeformers isolated from soil are variants selected from the mesophilic population during the process of elective culture, or whether they exist as such in the soil. At least a partial answer may be given

by comparison of the numbers of mesophilic and thermophilic sporeforming bacteria present in soil samples. The ratio of bacteria developing at 55–60 C on yeast agar plates to that of sporeforming bacteria growing at 35 C under similar conditions was found to be from 1/100 to 1/1,000, using various soil samples. Moreover, most of the thermophilic colonies contained *B. circulans* type bacteria, while *B. subtilis*, *B. megaterium*, and related forms were most common among the mesophils. It must be concluded therefore that either the frequency of variation of sporeformers fresh from soil is several orders of magnitude greater than that found with laboratory cultures, or that thermophilic sporeforming bacteria occur as such in the soil. There are natural environments favorable for their development, their spores are highly resistant, and some distribution of microbes from these natural sources must occur; hence, it would be rather surprising if no thermophils as such were found in soil.

5. *Classification of the thermophilic aerobic sporeforming bacteria.* Until quite recently there has been no useful system of classification of the thermophilic bacteria. Almost every investigator of the field has published his own names, and it is often not clear how many of the long list thus obtained may be synonymous. However, as knowledge of the genus *Bacillus* in general has increased, some order has been brought into the thermophils, while the latest developments eliminate much of the necessity for a separate classification of these organisms.

An important contribution to the knowledge of sporeforming bacteria capable of growth at high temperatures was made by Gordon and Smith (80). These workers assembled a collection of all the previously named cultures of thermophilic sporeformers which they could obtain (a surprisingly small collection, considering the number which has been described and the longevity of sporeforming bacteria), as well as some unnamed isolates from food products. The collection was further supplemented with cultures isolated by these authors (using nutrient broth or nutrient broth with glucose) and studied according to the methods developed by Smith *et al.* (155) for the mesophilic bacilli.

A number of the cultures were not thermophilic when received by Gordon and Smith, others were "recognized as members of species previously regarded as mesophilic", while the remainder fell into two distinct groups, one of which was identifiable as *B. coagulans* Hammer, the other as *B. stearothermophilus* Donk. These two were studied in detail and emended descriptions given. As a result of this work it is now possible readily to identify isolates falling into these groups. *B. coagulans* has been studied by several investigators in addition to Gordon and Smith (6, 17, 87, 93). All agree on its characteristics, and the organism is readily recognizable through its ability to grow in acid media and its lack of nitratase and proteinases. *B. coagulans* hence is not a taxonomic problem.

There no longer appears to be any reason to retain or establish separate species names for the other thermophilic sporeformers. As Gordon and Smith (80) pointed out, and as has been confirmed in this study, *B. stearothermophilus* is so much like *B. circulans* that they can be separated only on the basis of growth at 65 C. Now that variants of *B. circulans* have been obtained which are indistinguishable from authentic strains of *B. stearothermophilus*, it might seem

justifiable to regard the latter as a variety of *B. circulans*. However, since thermophilic variants of *B. circulans* can be obtained as readily as nutritional or antibiotic resistant mutants, and since such strains are not usually accorded varietal status, it seems best to speak merely of thermophilic strains of *B. circulans*.

The round-spored thermophils, which form a homogeneous and readily recognizable group, differing only in temperature range from the mesophilic *B. sphaericus* may similarly be considered merely as thermophilic strains of *B. sphaericus*. Even though in this case the conversion from one to the other has not been experimentally induced, their similarity is so close as to justify this terminology.

The thermophilic strains of the subtilis group do present a problem. In the first place, *B. subtilis*, as it is at present defined in Bergey's *Manual* (21), contains two types of bacteria which are in several ways quite different. The type culture, the so-called Marburg strain, is a strictly aerobic organism which has a maximum temperature of growth sufficiently high that it may almost be included among the thermophils if the definition of any organism growing at 55 C as a thermophil be accepted (92). The Marburg strain can reduce nitrate to nitrite and to ammonia, but does not form gaseous reduction products from nitrate (170). Associated with this type of organism under the name of *B. subtilis* are also the so-called Ford strain and others which resemble it, which a number of workers claim deserve the rank of a separate species (76, 92, 93, 170). The Ford strain is a facultative anaerobe which carries out an unusual type of sugar fermentation (24). Moreover, bacteria similar to the Ford strain have a lower maximum growth temperature than the Marburg type cultures (92), and they reduce nitrate to nitrous oxide and nitrogen (170) and give a positive lecithinase reaction (93) which the Marburg strain does not. Since these differences are greater than those used to separate *B. subtilis* from *B. pumilus* (the former reduces nitrate to nitrite and forms amylase, while the latter does not) (155), the claim that the strains of the Ford type should be separated from *B. subtilis* and given separate species rank under the name of *B. licheniformis* (Weigmann) Gibson appears well founded.

Of 20 thermophilic isolates of the *B. subtilis* group, 12 could grow anaerobically in glucose media, and also developed anaerobically with nitrate, forming gaseous reduction products. Five were strict aerobes and could not grow anaerobically with nitrate. The first group clearly corresponds to *B. licheniformis* and the second to *B. subtilis*. On the other hand, one culture which grew anaerobically with glucose did not do so with nitrate, while the most vigorous denitrifier in the group was incapable of anaerobic development with sugar. These latter cultures may be considered as *B. subtilis-B. licheniformis* intermediates.

Second, it must be emphasized that these names are only summaries for a set of characteristics and imply little about the ancestry of the organisms. Thermophilic bacteria resembling *B. subtilis* or *B. licheniformis* are obtained, not only from mesophilic cultures of these bacilli, but from cultures of *B. cereus* and *B. megaterium*. More work to determine the effect of this finding on the status of these two species would be desirable. Small-celled mesophilic variants of *B. megaterium* and *B. cereus* have been reported by Lehmann and Neumann (103)

and by Haag (82). Several types of sporeforming bacteria, previously regarded as distinct species, have been reduced to the status of varieties when techniques have been found for obtaining one as a variant form of the other. Examples of this are *B. mycoides* and *B. anthracis*, both of which are now regarded as varieties of *B. cereus*. The evidence is certainly not adequate to consider *B. megaterium* and *B. cereus* as varieties of *B. subtilis*, but it does raise a question concerning the relationship of these three organisms. It has been clear that the various members of Smith's morphological Group 1, the so-called "hay bacilli", have many properties in common; specification of the details of their relationship does not seem possible at present.

It may be concluded that thermophilic strains of almost any mesophilic *Bacillus* may occur and that there is little justification for a separate classification of the thermophils. A similar conclusion was recently reached by Gyllenberg (81a), who found that both his own thermophilic isolates and many of those described in the literature could be satisfactorily identified by using the current key to the mesophilic sporeformers. His isolates included both eurithermal and stenothermal strains identifiable as *B. subtilis*, others which could be called *B. cereus*, and some which resembled *B. circulans*. He considered it quite likely that further investigation would reveal close relationships between thermophilic and mesophilic sporeformers so that the grouping of the genus *Bacillus* into mesophils and thermophils for purposes of classification might well be abolished.

II. BIOCHEMICAL ACTIVITIES OF THE THERMOPHILS

1. *Decompostion of natural high polymeric materials.* As might be expected in a group of organisms which appears most conspicuously during microbial attack on masses of plant material, the earliest known biochemical activity of the thermophilic aerobic sporeforming bacteria was their ability to decompose natural high polymeric substances found in plants. The production of amylase and of proteinases by these microbes was recorded by almost every early worker (12, 27, 44, 45, 96, 98, 99, 109, 113, 133, 149, 152, 153); de Kruijff (98, 99) noted lipase as well. Several investigators have obtained thermostable hydrolytic enzyme preparations from the culture filtrates of thermophilic sporeformers (98, 99, 133, 139, 140); the properties of these enzymes have not, however, been studied in detail.

The breakdown of cellulose by thermophilic bacteria has been a popular subject of investigation. Under natural conditions, the decomposition of cellulose at high temperatures, as at lower ones, proceeds through the action of a complex association of microorganisms. Most workers who have attempted to isolate the organisms responsible for the primary attack on cellulose have reached the conclusion that the thermophilic bacteria which actually decompose cellulose are obligate anaerobes (164, 171). The aerobic and facultatively anaerobic sporeformers associated with the anaerobes have been assigned a secondary role (158, 164, 165). The study of McBee (110), who had indubitably pure cultures, leaves no doubt that there are obligately anaerobic thermophilic bacteria which can decompose cellulose.

Claims have also been made, however, for cellulose decomposition by aerobic

thermophils. The first of these stems from Kellerman and McBeth (90) and from Kroulik (97). The former, who reported the isolation of a pure culture of an aerobic thermophilic cellulose decomposer, mention it only in passing; while the statement of Kroulik is based on admittedly crude cultures. Since it is well known that in mixtures of bacteria anaerobic forms can develop under what appear to be aerobic conditions as witnessed, for example, by the appearance of *Clostridium pasteurianum* in the thin layers of liquid employed in enrichment cultures for *Azotobacter*, it is difficult to draw any conclusions on this point from experiments with crude cultures.

Slightly better evidence for aerobic cellulose decomposition was furnished by Coolhaas (44, 46). From crude cellulose fermentations he isolated thermophilic bacteria which grew on cellulose agar plates under aerobic conditions. Colonies on cellulose agar were surrounded by a clear zone, indicating dissolution of the cellulose. However, cultures isolated by successive platings on cellulose agar would not decompose cellulose when reinoculated into liquid medium. Addition of yeast extract or peptone to the liquid cellulose medium did not induce decomposition of the carbohydrate. This result has been interpreted as indicating impurity of Coolhaas' cultures (131); it seems, however, equally possible that it is due to the effect of changing physical conditions on the growth of the cellulose decomposing thermophils. It is perhaps not generally appreciated how much influence the physical set-up of an experiment has on the results obtained with thermophilic bacteria, especially when they are not permitted to live in a rich, complex medium but are compelled to synthesize all their cell material from one or a few compounds. To cite one example: the solubility of oxygen in water at 55 C is about one-half its value at 30 C, while, because of increased metabolism at high temperatures, the need of otherwise similar aerobic bacteria for oxygen is at least eight times greater. In addition, rapid evaporation from an exposed liquid surface at an elevated temperature may result in conditions at the surface very different from those obtaining at lower temperatures.

The importance of physical conditions for thermophilic cellulose decomposing bacteria was recognized by Murray (126), who reported aerobic growth of such organisms in atmospheres where the humidity was maintained at 98–100%, little or no growth when the humidity fell to 90% or less. He believed that many failures to obtain pure cultures of thermophilic aerobic cellulose decomposers were due, not to the anaerobic nature of the cellulolytic organisms, but rather to insufficient water vapor in the air. Similar effects of humidity on the growth of organisms have been observed with blue-green algae (3). Unfortunately, Murray gave no descriptions or other detailed information concerning the cultures which he isolated, nor did he study their mode of attack on cellulose. Other workers, also aware of the importance of physical conditions (63, 160), have claimed that anaerobiosis is necessary for the development of cellulose decomposing thermophilic bacteria.

Whether or not thermophilic aerobic cellulose decomposing bacteria can be isolated, the products obtained from crude fermentations of cellulose or cellulosic materials suggest that anaerobic cellulose breakdown is more important under

natural conditions. Hydrogen, carbon dioxide, ethyl alcohol, acetic acid, and occasionally butyric acid have been found as products of high temperature cellulose breakdown (26, 97, 136, 137, 140, 156, 164). These are the products formed by pure cultures of anaerobic cellulose fermenters (110, 132, 157). Thermophilic cellulose decomposition, like that occurring at low temperatures, is generally more vigorous with crude, mixed cultures than with pure ones (150). In one case (158), noncellulolytic bacteria associated with thermophilic cellulose fermenters have been isolated and, when mixed with the cellulose attacking form, shown to increase the rate of fermentation, probably because they removed inhibitory end products of cellulose decomposition.

Still less is known regarding the decomposition of other constituents of plant material by thermophilic bacteria. There is almost no literature on the breakdown by thermophils of such materials as hemicelluloses, pectin, lignin and chitin. Fred *et al.* (68) remarked that decomposition of hemicelluloses in plant materials at high temperatures proceeded parallel to that of the cellulose. Waksman *et al.* (173, 174) stated that at 75 C only the hemicelluloses of stable manure were attacked. Crude cultures capable of breaking down pentosans have been obtained here, but none which decomposes pectin or chitin. Bacteria develop in enrichment cultures containing these latter materials, but there is no evidence of breakdown of the polymer.

Several strains of the *B. circulans* group, isolated from contaminated agar, were found to possess the property of hydrolyzing this polysaccharide. When the oxygen supply of the bacteria was limited, the products of hydrolysis accumulated in the medium. The bacteria split agar to reducing sugars, leaving a residue which resisted further attack by these bacteria, but which could be hydrolyzed to reducing sugar with dilute acid. Further study of the process was handicapped by a lack of knowledge of the chemical composition and structure of agar; the results obtained here indicate that it is a much more complex substance than is generally assumed (137). In addition to galactose, small amounts of another hexose fermentable by yeast, probably fructose, and a relatively large amount of an unfermentable and unidentified reducing sugar were found, both on bacterial and on acid hydrolysis of agar. The presence of up to 40% of nonreducing material in agar hydrolyzates was also observed.

2. *Fermentation of carbohydrates.* Almost all of the thermophilic aerobic sporeforming bacteria can produce more or less acid from a wide variety of carbohydrates and related compounds. *B. coagulans* and some cultures of the *B. subtilis* and *B. circulans* groups convert sugars to acidic products under strictly anaerobic conditions; the other bacteria require at least small amounts of oxygen. It is a noteworthy property of the thermophilic bacilli that they break down sugars anaerobically without the production of any appreciable quantity of gaseous products. The only exceptions known to this rule are: an organism isolated by Laxa (101) from a sugar factory which produced gas in concentrated sugar syrups; and Coolhaas' *B. thermobutyricus* (44, 45) which, although capable of aerobic growth, fermented sugars in a manner suggestive of an anaerobe, producing CO_2, hydrogen, and butyric acid in large amounts.

The fermentation of glucose and other sugars by *B. coagulans* has been studied by several workers (6, 17, 87). All the strains used form principally D-lactic acid from sugars. Small quantities of ethyl alcohol, acetic acid, 2,3-butylene glycol, and its oxidation product, acetoin, are also produced.

Several investigators have determined in more or less detail the products of carbohydrate fermentation by thermophilic bacteria of the *B. subtilis* group. Laxa (102) reported formation of lactic acid from sugars by a thermophil which was probably of the *B. subtilis* type. Schardinger (152) found D-lactic acid and acetic acid among the products of starch fermentation by a thermophilic *B. subtilis*. Another isolate, probably also of the *B. subtilis* group, formed acetic acid, a little butyric acid, and D-lactic acid from dextrin.

The most complete fermentation analyses have been presented by Coolhaas (44, 45). His *B. thermobutyricus* appears morphologically, and, in many of its physiological characteristics, similar to thermophils of the *B. subtilis* group al-

TABLE 3

Fermentation of carbohydrates by Bacillus thermobutyricus

CARBOHYDRATE FERMENTED	GLUCOSE	SUCROSE	STARCH
Hydrogen	0.85	1.70	0.78
Carbon dioxide	0.66	1.68	1.35
Butyric acid	0.66	0.71	0.87
Acetic acid	0.21	0.22	0.27
Propionic acid	0.04	0.04	0.01
Lactic acid	0.62	0.22	0.005
Insoluble carbohydrate	0.06	0.01	0.06
% recovery of carbon	96.5	96.0	96.0

Figures given in moles per mole of sugar fermented.

though its fermentation characteristics were, as has been mentioned, unusual. Table 3 shows fermentation balances calculated from Coolhaas' data.

Havanto (85) isolated from spoiled bread an organism which he considered to be a thermophilic variant of *B. mesentericus* (now a synonym of *B. subtilis*). This bacterium fermented glucose to a mixture of formic acid, acetic acid, lactic acid, and ethanol. Neither carbon dioxide nor hydrogen was formed, nor was glycerol, which is a conspicuous product of glucose fermentation by most reported mesophilic *B. subtilis* strains.

Investigations of the fermentation of sugars by thermophilic bacteria of the *B. subtilis* group encounter the obstacle that it is difficult to get complete fermentation of the sugar without introducing into the medium large quantities of material which interferes with the analysis. Fermentation proceeds vigorously in a complex medium with 1–2% glucose, for example, but the ratio of glucose fermented to the yeast extract or peptone which is added to obtain good anaerobic growth and complete fermentation is not high enough to exclude the possibility that an appreciable portion of the fermentation products arises from non-carbohydrate constituents of the medium. Nor is it possible to circumvent this diffi-

culty by working with resting cell suspensions since, as will be shown later, a temperature of 55 C in a medium which does not contain the necessary constituents for growth will rapidly inactivate and kill thermophils of the *B. subtilis* group. Havanto (85) did manage to work with what he considered to be resting cells by means of a *tour de force*, employing several grams of bacteria to ferment a few grams of glucose. In view of the rapid autolysis which follows upon the death of cells of thermophilic *B. subtilis*, the interference from bacterial lysis products in this procedure was probably as great as that from yeast extract in growing cultures, and would be even more difficult to allow for.

Despite these difficulties, it has been possible to make reasonably complete analyses of the fermentation products formed from glucose by several thermophilic *B. subtilis* strains. By using relatively large inocula in a medium containing 0.5–1.0% glucose and 0.5% casein hydrolyzate, with either potassium

TABLE 4

Fermentation of glucose by thermophilic and mesophilic strains of Bacillus licheniformis (B. subtilis)

PRODUCT \ CULTURE	THERMOPHILIC		MESOPHILIC*		
	16-3	18-1	5	299	198
Carbon dioxide	0.226	0.380	1.12	1.02	0.046
Formic acid	0.040	0.069	0.19	0.082	0.242
Acetic acid	0.226	0.158	0.006	none	0.070
Ethanol	none	0.107	0.216	0.122	0.046
Lactic acid	0.969	1.04	0.248	0.385	1.37
2,3-butanediol	0.385	0.403	0.483	0.518	0.081
glycerol	none	none	0.464	0.413	none
% recovery of C	87.5	95.4	99.5	97.8	86.4
O/R index	0.87	0.58	1.00	0.96	1.08

* Data of Blackwood *et al.* (24).

Figures in moles per mole of sugar fermented.

phosphate or sodium bicarbonate as buffer, complete fermentation of the glucose was achieved in 8 hours at 55 C, with introduction of a minimum quantity of interfering materials. Table 4 presents analyses of such fermentations by two thermophilic *B. subtilis* strains. At the time of these analyses, strain no. 16-3 could grow at 25 C, as well as at 55–60 C, while strain no. 18-1 could not develop below 40 C. Analyses by Blackwood *et al.* (24) of the products of glucose fermentation by some mesophilic Ford type strains of *B. subtilis* are shown for comparison. It is evident that the glucose fermentation of the thermophils closely resembles that of those mesophilic strains which produce large amounts of lactic acid and relatively little glycerol.

It is, however, also apparent that there are anomalies in these fermentation balances. Not only is proportion of reduced products too high but also one-carbon compounds are deficient. The latter conclusion assumes that glucose is initially split into two 3-carbon fragments which are then transformed further

so that each C_2 product or C_4 compound derived by condensation of C_2 fragments should be accompanied by a C_1 product. Since the recoveries of carbon are also somewhat low, the possibility exists that the missing oxidized products and C_1 compounds might have reacted with the amino acid constituents of the medium to form products not detected by the usual methods of fermentation analysis. The deficiencies in both oxidation-reduction balance and C_1 compounds can be accounted for if it be assumed that the "missing" C_1 fraction has formed carboxyl groups which have not been estimated.

Fermentations carried out in the presence of radioactive carbon dioxide showed fixation of CO_2 in cell material and in amino acids found in the medium, which is in accordance with this explanation of the anomalous fermentation balances. The situation is so complex, however, that it seems preferable to consider the analyses as presenting a general qualitative picture of the thermophilic *B. subtilis* fermentation, leaving the resolution of the anomalies to later, more detailed investigation.

3. *Oxidative metabolism.* The difficulty of growing these organisms in chemically defined media with a single carbon source has prevented much progress from being made in the knowledge of the types of compounds which can be oxidized by the thermophilic sporeformers, while the pathways of oxidation of those compounds known to be utilized remain completely unkown. Two ambitious attempts have been made to survey the oxidative capacities of some thermophilic bacilli, an early one by Bardou (12), and a later work by Krohn (96). Both of these suffer from the handicap that the experiments were carried out in media containing high concentrations of peptone or similar complex nutrients in addition to the material presumably being studied, and that growth, rather than substrate disappearance, was the quantity measured. In such complex media it is difficult to distinguish between increased growth accounted for by utilization of the putative substrate and effects arising from, for example, the buffering capacity of the material added.

Some general conclusions on the oxidative abilities of the thermophils can be extracted, however, from these papers, from scattered reports in other works, and from observations during the present study. Sugars and polyhydric alcohols are usually readily oxidized, as are salts of lactic acid and the common C_4 dicarboxylic acids. Some types utilize citrate, as has already been pointed out in the discussion on characterization. Little information is available on utilization of fatty acid salts by pure cultures. Amino acids are usually excellent substrates for oxidation. Experiments with enrichment cultures suggest that thermophils can oxidize aromatic rings although it may not be a property widely distributed among them; phenol oxidizing cultures have been reported (61, 62). No information is available on the utilization of amines or of monohydric alcohols. Utilization of purines or pyrimidines has been observed only in enrichment cultures.

As far as these fragments of information extend, they indicate that the oxidative capacities of the thermophilic members of the genus *Bacillus* are at least as great as those of the mesophilic members. den Dooren de Jong (53) studied the dissimilative abilities of three representatives of the mesophilic aerobic spore-

forming bacteria, *B. vulgatus* (*B. subtilis*), *B. mycoides* (*B. cereus* var. *mycoides*), and *B. polymyxa*, together with those of a large number of other bacteria. The bacteria were grown on a simple mineral medium, containing agar and tapwater, which presumably took care of many growth factor requirements. The sporeformers could use for growth only a small number of the great variety of compounds tested; *B. subtilis* was the most versatile of the three bacteria tested. When glucose was present as a carbon source in the medium, however, a large number of the amino acids, amides, amines, and derivatives of urea, guanidine, and purines could serve as nitrogen sources for the bacilli; *B. polymyxa* was especially versatile with respect to the number of nitrogenous compounds it could utilize.

A similar survey of the capacities of thermophilic sporeformers appeared desirable, hence one strain of each group was tested by the auxanographic method for its utilization of various organic compounds. The strains used were selected for their ability to develop with a minimum number of growth factors and under the greatest variety of conditions. The basic solution with which the bacteria (grown on yeast agar at 55 C) were mixed contained: ammonium sulfate, 0.1%; K_2HPO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; yeast autolysate, 0.01%; and agar, 2%. No appreciable background growth was obtained with this quantity of yeast autolysate. Small bits of the compounds to be tested were placed as solids on the agar plates containing the bacteria, which were incubated at 55 C for up to 48 hours. The results are shown in table 5, together with some of den Dooren de Jong's results for the mesophilic sporeformers on the same compounds. In general, they confirm previous conclusions, especially in showing the limited dissimilatory versatility of *B. coagulans*. Study of a much greater number of isolates of each type will be required before it can be stated to what extent the oxidative patterns obtained are characteristic of each group.

4. *Transformations of nitrogenous compounds.* The most conspicuous conversions of nitrogenous compounds effected by thermophilic aerobic sporeforming bacteria are proteolysis, ammonification, and denitrification. The abilities of the thermophils of the various groups to carry out these processes have already been discussed.

Oxidation of ammonia to nitrite by cultures of thermophilic sporeformers has been reported (34), but the process was not studied in detail, and no other isolations of thermophilic nitrifiers have been made.

de Kruijff (98, 99) observed growth of thermophilic bacteria in nitrogen-free media but did not obtain sufficient nitrogen fixation for the increase in N content to be detectable by Kjeldahl analysis. Later, H. Pringsheim (138) reported nitrogen fixation by crude cultures of thermophilic bacteria. Three to six mg N was fixed per gram of sugar utilized, an amount appreciably less than that fixed by *Azotobacter* spp at ordinary temperatures. He reported no attempt to isolate the causative organism in pure culture nor gave any description. Enrichment cultures for nitrogen fixing thermophilic bacteria during the present investigation have been unsuccessful.

It must be concluded either that thermophilic bacteria which can carry out

TABLE 5

Suitability of various organic compounds for aerobic growth of some thermophilic and mesophilic sporeforming bacteria

ORGANISM / SUBSTRATE	THERMOPHILS				MESOPHILS		
	B. circulans 7-2	*B. sphaericus* 112	*B. coagulans* 139	*B. subtilis* 18-2	*B. vulgatus*	*B. mycoides*	*B. polymyxa*
glucose	+	+	+	+	+	+	+
galactose	+	+	−	+	±	±	+
arabinose	−	−	−	−	+	±	+
maltose	−	+	±	+			
lactose	−	−	−	−			
mannitol	+	+	+	+	+	±	+
acetate	+	+	±	+	±	±	−
lactate	+	+	±	+	+	−	−
succinate	+	+	−	−	+	−	−
tartrate	+	+	+	+	−	−	−
laurate	±	+	±	+	−	−	−
glutarate	±	−	−	−	−	−	−
glutamate	+	+	−	+	+	−	−
glycine	+	−	−	−	−	−	−
alanine	+	+	−	+	+	−	−
arginine	+	+	−	+	+	+	−
histidine	+	+	−	+	+	−	−
serine	−	−	−	−			
benzoate	−	−	−	−	−	−	−
p-OH benzoate	−	−	−	−	−	−	−
phenol	+	−	−	−	−	−	−
phenyl acetate	+	−	−	±			
tyrosine	±	−	−	−	−	−	−
cinnamate	−	−	−	−			
glucosamine	−	+	−	+	+	±	+
acetamide	−	−	−	−	±	±	−
glutamine	−	+	−	−			
triethylamine	−	−	−	−	−	−	−
ethylamine	−	−	−	−	−	−	−
urethan	−	−	−	−			
nicotinamide	−	−	−	−			
uracil	−	−	−	−			
guanine	−	−	−	−	±	−	±
indole	−	−	−	−	−	−	−
tryptophan	−	−	−	−			
proline	+	+	−	−			

transformations, other than denitrification, of inorganic nitrogen compounds are rare, perhaps even to the point of nonexistence, or that the conditions for obtaining their development are not understood.

5. *Nutritional requirements.* Most of the numerous investigators who have cultured thermophilic bacilli have employed complex nutrients for this purpose; few have attempted to obtain growth of the organisms in media of known chemical composition. Much of the work was done at a time before the development of present concepts of microbial nutrition, and various possible growth factor requirements have been hidden under statements that the thermophilic bacteria require organic nitrogen, or even that they are "peptone organisms". Such statements have arisen from the fact that few thermophilic sporeformers can develop in a simple mineral medium with ammoniacal nitrogen and a single carbon source.

Andersen and Werkman (6) investigated the nutritional requirements of a strain of *B. dextrolacticus* (*B. coagulans*). It required either thiamin or riboflavin (but not both), as well as a factor present in hydrolyzed casein and in an ether extract of acidified yeast extract. The latter factor could be replaced by glutamic acid, arginine, threonine, or cystine. Cleverdon *et al.* (40, 41) examined the nutritional requirements of a strain of *B. coagulans* and of twelve cultures of stenothermal thermophilic aerobic sporeformers (presumably of the *B. circulans* group). All of these cultures could be grown in a casein hydrolyzate–glucose medium (supplemented with tryptophan and cystine) when biotin, niacin, and thiamin were added. All three vitamins were essential for growth. Other investigators who have studied *B. coagulans* have found different vitamin requirements for this organism. Knight and Proom (93) found that their strains needed only thiamin and biotin, while all but one of the isolates studies here grew with only biotin and riboflavin. It must be concluded either that the composition of the basal medium influences the vitamin requirements of this organism, or that different strains have different demands.

The first experiments on the nutrition of the collection of cultures used in the present study suggested somewhat complex growth factor requirements. Growth in a simple medium consisting of: ammonium sulfate, 0.1%; K_2HPO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; and 0.1–0.5% of glucose, glycerol, sucrose, mannitol, lactate, acetate, succinate, or malate was at best very scanty at 55 C and not usually transferable. The majority of the thermophilic isolates did not grow at all at 55 C in these media. However, some of the cultures which could grow at low as well as at high temperatures would grow in such simple media at 35–40 C. Glucose, lactate, and malate were the best carbon sources for growth at the lower temperature. Often, growth at 55 C was extremely poor even when the medium was supplemented with a mixture of all the usual vitamins, with an amino acid mixture, or with both, although addition of 0.1% yeast autolysate always permitted good growth.

Addition of 0.3% monosodium glutamate to the simple medium with glucose permitted growth at 55 C of four strains of the *B. circulans* group, three of *B. coagulans*, one of *B. sphaericus*, and one of *B. subtilis* without addition of vitamins or other amino acids. Further supplementing with 0.1% casein hydrolyzate stimulated growth of the cultures developing in the simple medium and made possible development of one more strain of *B. sphaericus*, two more of *B. coagu-*

lans, and two more of *B. subtilis*. Addition of a vitamin mixture instead of casein hydrolyzate made the medium suitable for three more *B. circulans* cultures, as well as one of *B. sphaericus*. When both vitamins and casein hydrolyzate were added, all the cultures tested grew and were transferable in the glucose-glutamate medium although growth with some strains remained slight. Attempts to determine more closely the vitamin and amino acid requirements of several selected strains led to inconsistent results. The action of glutamate in promoting growth at high temperatures recalls the case of *Escherichia coli* (175) which does not require growth factors at 35 C but needs glutamic acid to grow at 44 C.

Recent work (11) has indicated that the growth factor requirements of the thermophilic bacilli are in reality quite simple, that they do not need extensive vitamin and amino acid supplements, and that much past difficulty in growing these organisms in defined media must be traced to the use of an inadequate mineral base and, possibly, of unsuitable carbon sources or substrates at too low a concentration. The conditions of life at high temperatures are more delicately balanced than at low ones so that conditions which would be only mildly inhibitory at ordinary temperatures, such as the production of small amounts of acid or slow utilization of the carbon source supplied, can at 55–60 C either make growth impossible, or make it necessary to furnish many growth factors and building blocks for cell material.

Using a basal medium which contains several carbon sources and a richer complement of mineral nutrients[1], we obtained growth with one thermophilic strain of *B. sphaericus* and two of *B. coagulans*. Addition of biotin and riboflavin to the medium made it suitable for growth of a number of thermophils of all types. With most of these, excellent growth also resulted, surprisingly, if instead of the vitamins a large quantity of calcium ions (12 mg %) was added to the culture solution.

In a number of cases, notably with representatives of the *B. sphaericus* group, development in this medium was limited by the acidity resulting from the metabolism of glycerol. Substitution of Ca gluconate for glycerol in the medium made it an excellent growth substrate for most of the bacteria tested. Out of 48 strains investigated, only four of *B. circulans*, one of *B. coagulans*, and two of *B. subtilis* failed to grow in the gluconate medium. With most of the others, growth was heavy and comparable to that obtained in natural complex media.

In addition to materials which are necessary if growth is to occur at all, a great many substances have been observed to stimulate growth of the thermophilic bacteria. Media designed for optimal growth, therefore, may become quite complex (11), even though it is clear from the development of many strains in simple media that the thermophilic aerobic sporeforming bacteria, considered as a group, have extensive synthetic powers. There are also many instances in which it is possible to substitute one growth promoting substance for another. Analysis

[1] $(NH_4)_2H$ citrate, 0.25%; KH_2PO_4, 0.02%; $MgSO_4 \cdot 7H_2O$, 0.03%; sodium succinate $\cdot 6H_2O$, 0.3%; monosodium glutamate, 0.4%; Ca^{++}, 0.5 mg %; Mn^{++}, 2.0 mg %; and glycerol, 0.5%; with one ml per 100 of trace metal solution, consisting of 2.5 g ethylene diamine tetraacetic acid (Versene), 11.0 g $ZnSO_4 \cdot 7H_2O$, 4.61 g $MnSO_4 \cdot H_2O$, 2.1 g $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 0.477 g $CoSO_4 \cdot 7H_2O$, 0.0571 g H_3BO_3, 0.0078 g $CuSO_4 \cdot 5H_2O$, water 1 liter (11).

of some of these examples of substitution may lead to knowledge of the relations between different growth factors. However, as will be shown later, thermophilic bacteria expend a great deal of their synthetic powers in overcoming the destructive effects of heat. Stimulation of growth by any one of a number of substances could thus indicate merely that metabolic leaks may be plugged at any one of a number of spots, leaving more energy and synthetic capacity available for use on other pathways, and need not mean that the alternate materials lie on the same synthetic path.

Unfortunately, the synthetic media so far developed which permit good growth contain rather large amounts of amino acids and salts of carboxylic acids so that they are of little use in overcoming the difficulties already discussed in connection with analyses of the fermentations of carbohydrates by thermophilic sporeformers.

III. FACTORS WHICH MAKE THERMOPHILY POSSIBLE

Perhaps the most interesting problem raised by the thermophilic sporeformers is how they are able to develop under such apparently unfavorable conditions. The evidence so far obtained indicates that no one answer to the question can be given. The external environment of the cells, structural features of the organisms, including structural modifications on a molecular level, and dynamic effects of metabolism in preventing or repairing thermal damage all play a part.

1. *The external environment.* It has often been noted that the thermal resistance of microorganisms depends upon the environment in which they are placed; the most obvious example of this is the well known fact that bacteria can survive in a dry condition heat which is lethal when they are wet. Examples indicating an effect of the chemical composition of media used for growth or preservation of microorganisms upon the heat resistance of the organisms are readily found. Nakamura (127) in 1897 reported that beer yeast, which completely lost its ability to ferment on heating for 30 min at 50 C in water or 10% sucrose solution, retained its fermenting capacity if heated in a sugar solution containing the minerals necessary for growth, or in meat extract. The ability to reproduce was lost in all cases. Göbel (78) obtained higher survival of *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*) in bouillon than in physiological salt solution when the two suspensions were exposed to the same temperature. Richet *et al.* (146) increased the maximum growth temperature of a lactic acid bacterium from 36–37 C to 41–42 C by cultivation in the presence of 3.4% KCl.

Casman and Rettger (35) failed to find any protective effect of hypertonic solutions, such as 20–40% peptone, 10–15% NaCl, and 30–35% lactose, on a number of organisms of the *B. subtilis* group. On the other hand, Baumgartner (15, 16) reported that 0.5 M solutions of glucose, galactose, lactose, maltose, sucrose, mannitol, and glycerol protected *E. coli* from thermal destruction. The sugar solutions exerted their protective effect only if they were filter-sterilized; sugars autoclaved in buffer or nutrient broth lowered the thermal resistance. Anderson *et al.* (7) found that glucose and sucrose increased the heat resistance of *B. coagulans*, while NaCl and several organic acids decreased it.

van Halteren (83) has shown that yeast is protected against the effects of

heat by concentrated solutions of various substances. Yeast heated for one hour at 44 C in the presence of 1 or 2 M NaCl, 1, 2, or 3 M glucose, or 30% sucrose showed little, if any, decrease in respiration and was able to assimilate nitrogen immediately on being returned to 37 C, while if the yeast was heated in a dilute medium containing glucose, minerals, and succinate buffer, oxygen uptake was markedly reduced, and nitrogen assimilation did not proceed for several hours after the period of heating.

The explanation of these effects is not always clear, but two possibilities may be envisaged. Suitable media may increase heat resistance by providing favorable conditions for active metabolism so that the organism is better able to repair damage done by heat, or they may aid growth at high temperatures because they contain substances which protect proteins from thermal denaturation. The first effect may readily be observed with thermophilic bacteria of the *B. subtilis* group. Unless these bacteria are placed in an environment which permits active metabolism, their thermal resistance is not noticeably greater than that of analogous mesophilic organisms (2).

As to the second possibility, *i.e.*, the effects of sugars, neutral salts, and indifferent proteins in preventing enzyme inactivation, a few examples dealing with bacterial enzymes may be cited. Virtanen (172) found that the proteinase of *Bacillus fluorescens-liquefaciens* (*Pseudomonas fluorescens*) is not inactivated by boiling for 10 minutes in a medium containing additional soluble protein, but is readily destroyed at 60 C in a protein-free medium. Bacterial lipases behaved similarly. Maas (107) recently found that a mutant of *E. coli* which requires pantothenic acid for growth above 30 C contains an extraordinarily thermolabile pantothenate synthesizing enzyme. This enzyme is rapidly inactivated even at 25 C, but can be protected by sugars or neutral salts.

The effect of divalent ions on thermal resistance deserves more investigation. The decrease in growth factor requirements of some thermophilic sporeformers in media containing relatively high concentrations of Ca or Mg ions is suggestive of a protective effect, particularly when considered together with the finding that higher calcium content is one of the few chemical differences which have been found between bacterial spores, which are the most thermoresistant living objects known, and vegetative cells (49). In addition to its effect on the thermophilic sporeformers, calcium has been observed to increase the thermal resistance of *Paramecium* (38). It has also been noted that the bacteriophages of *E. coli*, which are notably thermoresistant in a suitable medium, as indicated by the report that the heat resistance of a bacteriophage active against thermophils at high temperatures (95) was similar to that of coli phage, are very sensitive to the effects of heat unless divalent ions are present in the medium (106).

2. *Structural modifications of the cell.* One generalization which may be made about thermophilic microorganisms is that they are usually small in size, at least in the sense of having slender cells. Several years ago Lamanna (100) pointed out that size decreases as temperature range for growth rises in the genus *Bacillus*. The point is further emphasized by the finding that the thermophilic variants of *B. cereus* and *B. megaterium*, unlike their parents, are characterized by slender

cells. A survey of the members of the genera *Phormidium* and *Oscillatoria* described by Copeland (47) in his monograph on the Yellowstone thermal Myxophyceae indicates that the same rule holds among the blue-green algae; those inhabiting hot waters are, almost without exception, small-celled types (*cf.* table 6). Thermophilic actinomycetes are, like their mesophilic relatives, of small diameter (105). Only among the fungi does it seem possible for cells 10 μ or more in diameter to exist at high temperatures (130). It is possible that this correlation between small diameter and ability to grow at elevated temperatures is coincidental. If it is considered significant, then it follows that, since cells of small diameter have a greater ratio of surface to volume than do larger cells,

TABLE 6

Comparison of diameters of mesophilic and thermophilic members of the genera Phormidium and Oscillatoria found in Yellowstone Park

FORMS GROWING AT 70 C AND ABOVE:	DIAMETER
	μ
Phormidium geysericola	0.4–0.6
Phormidium bijahensis	0.3–0.5
Oscillatoria filiformis	0.4–0.5
FORMS NOT FOUND ABOVE 40 C:	
Phormidium yellowstonense	6–8
Phormidium fragile	1.2–2.3
Phormidium foveolarum	*ca* 1.5
Phormidium luridum	1.7–2.0
Oscillatoria limosa	11–23
Oscillatoria anguina	6–8
Oscillatoria princeps	29–42
Oscillatoria sancta	10–20
Oscillatoria lacustris	5–7
Lscillatoria amphibia	2–3
Oscillatoria amoena	2.4–5
Oscillatoria splendida	2–3
Oscillatoria boryana	6–8

and since a large surface/volume ratio should be favorable for the rapid movement of substrate into and waste products out of the cell but unfavorable for the static conservation of material, rapid exchange of materials with the environment is of importance for organisms developing at elevated temperatures.

Although visible structural features of the cell might thus be presumed to have some importance for thermophily, much more attention has been given to what may be considered as morphological adaptations on the molecular level, such as changes in protein configuration resulting in an increased stability at high temperatures. Quite early in the investigation of thermophilic microorganisms it was found that hydrolytic enzymes resistant to high temperatures could be separated from the culture filtrates of thermophilic bacteria. Oprescu (133) obtained an

amylase preparation which was still active after heating for a half hour at 85 C although the proteinase activity of his filtrates was lost during heating for one hour at 60 C. Pringsheim (139, 140) studied a cellulase, from thermophilic cellulose fermenting bacteria, which hydrolyzed cellulose to cellobiose over the temperature range 20–70 C. These findings lent support to the view, which seems at first glance almost obvious, that the proteins of thermophilic microorganisms must be in some way modified so that they are not destroyed by the high temperatures at which the organisms grow.

As knowledge of enzymes and of microbial biochemistry increased, it became evident that extracellular hydrolytic enzymes need not be reliable indicators of the thermostability of the essential intracellular proteins of microorganisms growing at high temperatures. Attention then turned to the respiratory enzymes. Casman and Rettger (35) and Edwards and Rettger (60) measured the activities (in intact resting cells) of catalase, peroxidase, indophenol oxidase, and succinic dehydrogenase at various temperatures, using an experimental material several mesophilic members of the *B. subtilis* group and unidentified thermophilic organisms which were stated to be similar to *B. subtilis*. They demonstrated a general correlation between the maximum temperature of growth of the bacterium and the maximum temperature of activity of the above mentioned enzymes, but the exceptions were too numerous to lend very convincing support to the thesis that ability to grow at elevated temperatures depends upon altered temperature characteristics of these oxidative enzymes. Activity of some of the enzymes (notably peroxidase) could be demonstrated at temperatures above the maximum for growth of the bacteria; with others enzyme activity was suppressed by temperatures at which the bacteria grew well.

Militzer and his co-workers (74, 108, 115, 116, 117) have furnished good evidence for the presence of thermonstable respiratory enzymes in one thermophilic bacterium. From a strictly thermophilic organism tentatively identified as *B. stearothermophilus* (*B. circulans*) this group has obtained thermostable cell-free preparations of malic dehydrogenase, cytochrome oxidase, cytochrome b, cytochrome c, succinoxidase, aldolase, and apyrase. Most of these enzymes are found together in a particulate "red fraction" which appears to constitute a morphological entity within the cell, but a soluble apyrase has been prepared, indicating that combination in a particle is not necessary for thermostability of the proteins from the thermophil. It may, however, be worthy of note that the only thermostable enzymes procured in soluble form are hydrolytic.

Possession of enzymes which are less susceptible than usual to the effects of heat is undoubtedly a factor which contributes to the ability of some bacteria to grow at high temperatures, yet there is evidence that thermostable enzyme protein, at least for the usual respiratory enzymes, is not requisite for growth in the temperature range of thermophilic bacteria. Gaughran (69, 72) has studied the effect of temperature on the activity of the complete respiratory system and of catalase, cytochrome oxidase, and several dehydrogenases from five strains of stenothermal thermophils. These bacteria were not identified nor were their temperature ranges given beyond the statement that they did not proliferate at or below 37 C. Since most thermophilic bacteria with such an elevated minimum

temperature have a maximum temperature for growth of 65–70 C, it may be inferred that this was true for Gaughran's organisms. Using bacteria of this type, curves for the activity of all the enzymes studied showed appreciable inactivation (rate decreasing with time) at temperatures ranging from 37 to 50 C, where the bacteria grew well.

Similar effects have been observed with thermophils of the *B. subtilis* group (2). Measurements of the activity of the entire respiratory system in intact resting cells, and of catalase in dry cell preparations, both showed rapid inactivation at 55 C, even in a bacterium which had a temperature range for growth of 40–65 C. Moreover, bacteria which grew well at 55 C died at this temperature when incubated without nutrients, the rate of death being comparable to that of a mesophilic *B. subtilis* under the same conditions.

Comparative measurements of the thermal stability of the malic dehydrogenase of dried cells of thermophils of the *B. subtilis* and of the *B. circulans* groups have shown that the enzyme of the *B. circulans* thermophils is markedly thermostable, in agreement with the findings of Militzer *et al.*, while the corresponding enzyme in thermophils of the *B. subtilis* group is rapidly inactivated at 65 C. These results are shown in figure 1. The greater stability of physiological characteristics in the *B. circulans* group, as compared to the *B. subtilis* type thermophils, may well be a consequence of their complement of thermostable respiratory enzymes. Even in the *B. circulans* group, however, thermolabile enzymes are found. Militzer *et al.* (117) have recently examined the pyruvic oxidase from the same thermophilic bacterium which has yielded a number of thermostable enzymes and found the pyruvic oxidizing enzyme to be thermolabile outside the cell, although fairly stable in the intact organism.

Certain workers (18, 67, 70, 135, 162, 163) have emphasized the role played by the degree of saturation of the protoplasmic lipids in determining the temperature range for growth of an organism. Measurements have shown that an organism, whether a bacterium or an animal, grown at a high temperature has more saturated fatty acids in its lipids than one grown at a low temperature (18, 135, 163). This result has been interpreted in terms of the need for maintaining a certain protoplasmic viscosity if vital processes are to continue. While the fact of alterations in lipid composition appears well established, its meaning for the functioning of the organism is at present obscure. Experiments with higher animals indicate that the degree of saturation of the lipids varies in one and the same organism according to the external temperature during growth, even in organisms with a presumably constant body temperature (18, 67).

3. *Dynamic factors.* Since it has been shown that some thermophilic bacteria can grow at high temperatures without notably thermostable respiratory enzymes, evidently ability to counteract the destructive effects of heat must be important in permitting growth of thermophilic microorganisms. It can readily be shown that even in those thermophilic bacteria which contain thermostable enzymes cell material is destroyed during growth at high temperatures, and that active metabolism is necessary to keep the proportion of viable cells at a high level.

In contrast to mesophilic bacteria, which release peptides and amino acids into

the medium only after rapid growth has ceased (141), cultures of thermophilic bacteria contain appreciable amounts of extracellular material which reacts with ninhydrin even during the early phases of growth. Paper chromatography of

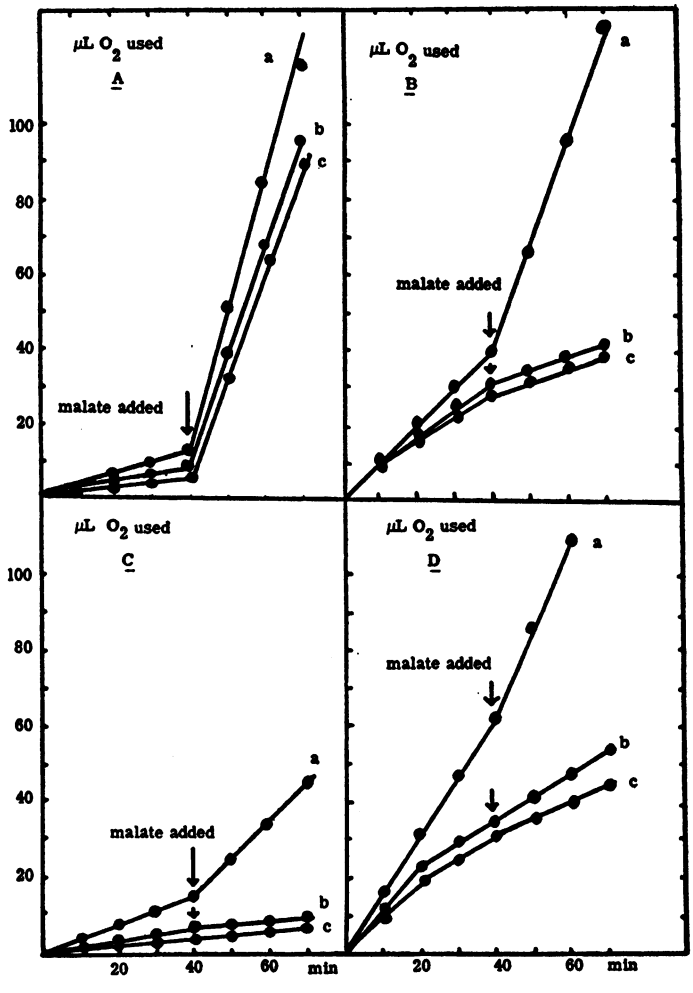

FIG. 1. Malic dehydrogenase activity of dried cells. Measured at 30 C, under the conditions described by Militzer *et al.* (115).

A. Thermophilic *Bacillus circulans*, strain 7-2. 20 mg dry cells per vessel.

B. Thermophilic *B. licheniformis*, variant of Ford strain S8. 80 mg dry cells per vessel.

C. Mesophilic *B. circulans*. 20 mg dry cells per vessel.

D. Mesophilic *B. circulans*. 80 mg dry cells per vessel.

Curves a, unheated; b, heated 15 min at 65 C; c, heated 30 min at 65 C.

culture filtrates taken during the logarithmic growth phase shows that they contain a complex mixture of amino acids and material of higher molecular weight which yields amino acids on hydrolysis.

Perhaps the clearest evidence for the necessity of active metabolism for maintenance of thermophilic organisms comes from a study of the kinetics of growth and death at high temperatures. Few data on this subject are in the literature, and since most of them were obtained under more or less unfavorable physical conditions for growth, they are of little value in indicating the rates of growth and death of thermophilic bacteria in a favorable environment. They do, however, serve as good illustrations of the extent to which death and destruction of cells can dominate the growth pattern in cultures at high temperatures.

The principal factor which has been overlooked or underestimated by previous investigators of the kinetics of growth of thermophilic bacteria is their extraordinarily high need for oxygen, coupled with the increased difficulty of supplying this gas fast enough at elevated temperatures.

Tanner and Wallace (161) grew thermophilic sporeformers in thick layers of liquid broth without aeration. The oxygen starvation thus resulting lengthened the measured generation time at 55 C to about 24 hours. A slightly better experimental arrangement was used by Hansen (84), who passed air over the surface of his culture solution. He observed a generation time at 55 C of *ca.* 16 minutes, using as experimental material a facultatively thermophilic organism belonging to Cameron and Esty's Group 80 (32). From their description of this group, and from the limiting pH of 4.7 for growth of the bacterium, it may be tentatively identified as *B. coagulans*, though it might be an especially acid resistant strain of the *B. subtilis* group. Hansen used a rather concentrated complex medium and did not, in most of his experiments, neutralize the acid formed by the bacteria from the glucose in the medium. His data on growth rate as a function of temperature are extremely irregular, probably owing to the combined and complex influences of several individual factors, *e.g.*, temperature, oxygen supply, and acidity, on the metabolism of the bacteria. Another complicating factor is the rapid death and lysis of vegetative cells in cultures of thermophils, especially when growth has come to a standstill. This very fact may entail that thereby favorable conditions for growth are once more established so that several cycles of growth and death may take place in one and the same culture. Such behavior, observed during the present study in cultures of thermophilic *B. subtilis* strains, is also illustrated by Hansen's curves.

Tanner and Wallace and also Hansen measured only the number of viable cells (by plate counts) present in their cultures. Imšenecki and Solnzeva (88) measured both the total number of cells (by direct microscopic count) and the number of viable cells. These investigators were also the first to recognize that oxygen lack may severely restrict growth of thermophilic microorganisms. Comparing growth in 1 cm thick layers of unstirred liquid with that in aerated cultures, they found that not only was growth more rapid in the latter, but also the total cell crop was much larger. They concluded that limitation of growth in cultures of thermophilic bacteria is very probably due to exhaustion of the oxygen supply. After reaching a maximum, the number of viable cells declines rapidly. Figure 2, plotted from their data, compares the numbers of total and of viable cells during growth of an eurithermal *Bacillus* sp at 60 C.

Since the results of Hansen and of Imšenecki and Solnzeva were obtained with eurithermal thermophils, and since organisms of this type have not been found to contain markedly thermostable proteins, it appeared of interest to investigate the growth and death of a bacterium of the *B. circulans* group, which grows only at high temperatures, and contains a thermostable respiratory enzyme system, to see if similar phenomena of rapid death occur with this type of bacterium. The organism chosen, strain 7-2, is capable of rapid growth in a medium containing minerals and sodium glutamate. Addition of a vitamin mixture and of

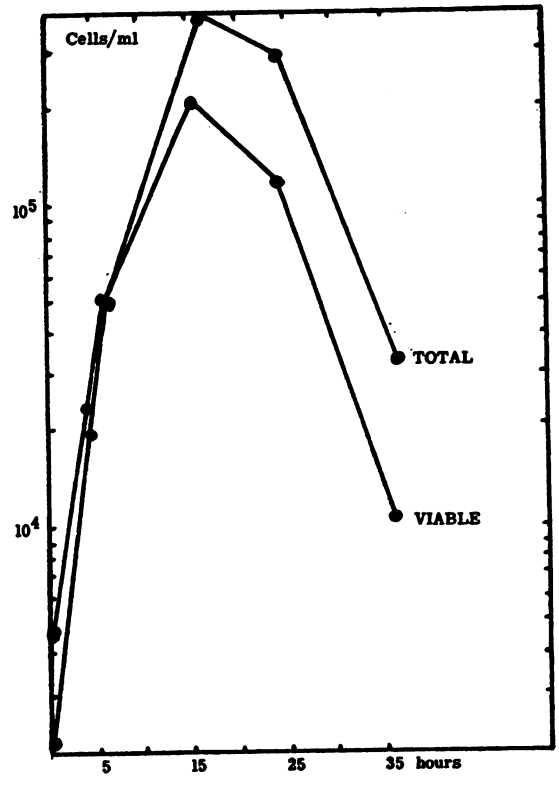

FIG. 2. Growth curves of an eurithermal *Bacillus* sp at 60 C, according to Imšenecki and Solnzeva (88).

casein hydrolyzate to the medium did not accelerate growth. The great demand of thermophilic bacteria for oxygen was clearly shown by the behavior of this strain; its growth rate was decidedly increased by aeration (using air saturated with water vapor at the temperature of the water bath) even in the few mm thick layer formed by placing 20 ml of medium in a one liter flask.

The apparatus is shown in figure 3. Growth was followed both turbidimetrically and by direct cell counts in a Petroff-Hauser chamber. Determination of the number of viable cells in a culture at high temperatures presents a problem which has not been recognized by earlier workers. If, as will be shown presently, there is

a high mortality even during growth of thermophilic bacteria, then determination of the number of viable cells by plating may be expected to give results which are too low, because of death of some cells, viable at the time of sampling, before they can develop into colonies. For this reason, viable cell numbers were determined not only by plating but also by direct counting in the Petroff-Hauser apparatus in the presence of dilute methylene blue, considering as dead those cells which stained blue. This method has been criticized (28) as given a too high result for the number of nonviable cells since some which are capable of reproduction may take up the dye. Nevertheless, the proportion of viable cells determined by direct counting in the presence of methylene blue always exceeded that found by plating, indicating that an appreciable number of cells die during the process of making plates and fail to produce colonies. This result confirms the view that, even with a thermophil containing relatively stable enzymes, rapid death may occur at temperatures favorable for growth.

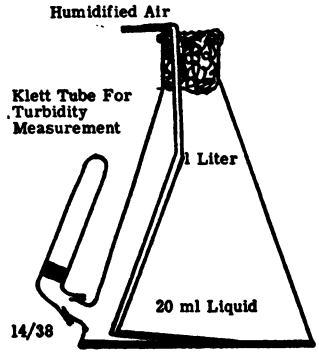

FIG. 3. Apparatus used for measuring the growth of thermophilic sporeformers.

The type of growth curve obtained with this bacterium is shown in figure 4. Turbidimetric and cell count measurements of growth were closely similar during the logarithmic phase but deviated when growth became slower. Although this organism does not show the spectacular lysis at the end of growth found with *B. subtilis* cultures at high temperatures, microscopic observation showed that at least some of the increase in turbidity observed while the cell count was falling was due to cellular debris. The possibility of a change in light scattered per cell must also be admitted; however, spores were not formed in the glutamate medium, nor did the cell shape or size change markedly during growth. Because of the experimental difficulty mentioned above, the figures for viable count must be considered as qualitative, or, at best, roughly quantitative, yet the general picture, especially the rapid drop in the proportion of viable cells when rapid growth ceases, is clear.

The maximum cell crop which can be produced as the result of rapid growth, such as is illustrated in the figure, is of the order of 10^8 cells per ml. Similar small crops were obtained by Hansen (84) and by Imšenecki and Solnzeva (88). It seems likely that this limit is imposed by the amount of oxygen which can be

supplied to the culture. Using rich, well buffered media, such as described in the section on growth factors, much greater cell crops can be obtained, but the rate of growth is far less after *ca.* 10^8 cells/ml have been formed.

It has previously been found that the yield of bacterial cells per unit quantity of nutrient becomes markedly less as the temperature increases (121). Such an effect would be expected if the organism must expend increasing amounts of energy in overcoming the destructive effects of heat as the temperature is raised. An extension of such measurements to the thermophilic sporeformers appeared of interest, but there were several experimental difficulties. To measure the crop per unit amount of nutrient, it is desirable to make sure that exhaustion of the substrate is the factor limiting growth. If a range of substrate concentrations can be found in which the crop is proportional to the quantity of substrate added,

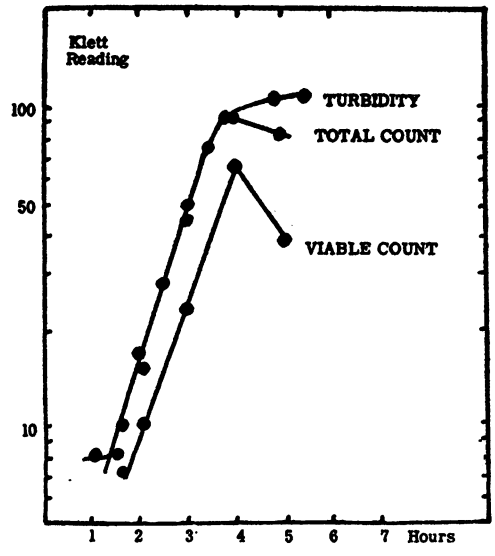

FIG. 4. Growth curves of thermophilic *Bacillus circulans*, strain 7-2, in glutamate medium at 60 C. A Klett reading of 100 corresponds to 3 x 10^8 cells/ml.

it may be concluded that this condition is fulfilled. Attempts to measure growth of the thermophilic *B. circulans*, strain 7-2, as a function of substrate concentration have shown that it is difficult to be sure that growth of thermophilic bacteria is limited by exhaustion of substrate, even with a bacterium like this, which develops in dilute solutions of a single oxidizable material. At 55–60 C, growth in the glutamate medium is approximately proportional to glutamate concentration over the range 0.05–0.1%. Above 0.1% glutamate other factors, presumably principally oxygen supply, limit the extent of growth, while in media containing less than 0.05% glutamate growth is uncertain and irregular. The data which have been obtained suggest that, unlike the *E. coli* studied by Monod (121), thermophilic bacteria require the expenditure of a measurable amount of energy for their maintenance, but the interrelation of the various factors which operate

to make growth of thermophils possible appears too complex to permit an unequivocal analysis of the effect of temperature on cell yield.

4. *Quantitative expression of the various factors.* In order to estimate the relative importance of the various static and dynamic factors for the development of different thermophilic microorganisms, a quantitative means of expressing their contributions must be found. It seems possible that such an expression can be achieved through a study of the kinetics of growth and death of bacteria.

It is well known that the rate of growth of microorganisms, like almost every other biological process, increases with temperature up to a certain point, after which it begins to decrease. The temperature dependence of the rising portion of the growth curve can be expressed by any one of a number of simple mathematical functions; of these, the one with the best theoretical fundation is the Arrhenius equation

$$dk'/dT = e^{-E/RT}$$

where k′ is the growth rate constant, T, the temperature, E, a quantity having the dimensions of energy, and R is the gas constant. As the temperature of most rapid growth is approached and passed, deviations from this equation appear.

The applicability of the Arrhenius equation to biological processes has been much debated, but the controversy appears now to be principally of historical interest although there may still remain a question as to the significance of the E (or μ) values (*cf.* 18, 48). Whatever the significance given to these figures, attempts to study complex processes, such as the growth of microorganisms, in such a way that they can be treated theoretically can lead not only to the refinement of experimental procedures but also to the recognition of complicating factors which might well go undetected otherwise. Many of the apparent abnormalities of biological systems may well be deceptions suggested by the inappropriate use of experimental data. If the ionization constant of phosphoric acid were formulated as $(PO_4^{---})/(H_3PO_4)$, the value of this ratio would change enormously at different hydrogen ion activities, and the dissociation of phosphoric acid might appear to be a process not amenable to simple theoretical treatment. The complexity of biological processes is such that we cannot expect to be aware of each of the reactants. Nevertheless, there is no reason to believe that such phenomena are intrinsically disorderly. A theoretical treatment may reveal abnormalities which, if studied in detail, can contribute toward a more complete understanding of complicating features.

For the growth of bacteria, there are few data which are complete enough to deserve theoretical treatment and which provide assurance that the investigator was measuring anything more significant than the rate of diffusion of oxygen into the solution. There are, however, some striking exceptions, such as the data of Monod (121) and of Johnson and Lewin (89a) on *E. coli*. The results of Slator (154) on *Lactobacillus delbrueckii* also appear worthy of treatment since this organism has primarily a fermentative metabolism and does not require oxygen for optimal development. Some data obtained on a thermophilic *B. circulans* strain are also considered suitable. This group of results has been selected from those available because the organisms constitute a series with increasing maxi-

mum temperatures of growth, and because in each case measurements were made of growth rates at various temperatures, including those at which deviations from the Arrhenius equation occur.

If the logarithm of the growth rate constant of any of these organisms is plotted against the reciprocal of the absolute temperature, according to the Arrhenius equation, it is seen that there is a range of temperatures over which a linear relation exists. Above this range, the rate of growth increases with temperature less rapidly than theory demands, and finally falls off sharply. If we assume that the rate of synthetic processes continues to increase exponentially with temperature, and that the falling off of the net growth is due to the increasing role played by destructive reactions as the temperature increases, then it is possible to calculate the temperature dependence of the destructive processes by extending the linear portion of the growth curve and computing the difference between this projection and the observed rate of growth. It is found that the curves for death rate vs. 1/T determined in this manner are also exponential. The temperature relations of growth and death for the bacteria considered here are shown in figure 5.

It appears reasonable to consider the temperature coefficient of growth as a measure of the role of dynamic processes in permitting growth at high temperatures, while the temperature coefficient of death may be related to the inherent stability or instability of the cellular constituents. It is tempting to suggest that *L. delbrueckii* can grow at a higher temperature than *E. coli* principally because of its greater temperature coefficient of growth, and that *B. circulans* depends to a large degree on its lower temperature coefficient of death, but more extensive and more accurate data will be needed before much reliance can be placed on these interpretations.

Another important factor demands evaluation. It will be noted that the rates of growth and death differ not only in their temperature coefficients but also in their magnitude at a given temperature. According to the theory of absolute reaction rates (which seems applicable at least to the destructive reactions since it has been applied successfully to protein denaturation (65)), the rate of a chemical reaction depends upon an exponential factor $e^{-\Delta H/RT}$ which gives the temperature coefficient, and on another factor which contains an entropy change. In the hydrolysis of polypeptides the entropy factor has been related to relatively small structural differences between the peptides.

In the foregoing discussion, emphasis has been placed on the role of synthetic processes in permitting growth at high temperatures. The available evidence suggests that the dissimilatory enzymes which for obvious reasons of accessibility have been most commonly studied are not the limiting factors for growth at high temperatures. The respiratory enzymes of thermophilic bacteria have been found to differ from those of mesophilic forms only in that, in some instances, they have an increased thermal stability. Even this difference is not obligatory. The fact that many thermophilic bacteria will not grow at ordinary temperatures while their respiratory enzymes may readily be observed to function under these conditions indicates that these enzymes cannot be the significant factors that control the growth of thermophils.

Consequently, it appears necessary to search elsewhere for factors which permit growth of bacteria at high and prevent growth at low temperatures. A possible direction in which to proceed is suggested by the studies of Rahn and his co-workers (143, 144, 145). These investigators have found that when bacteria are exposed to various destructive agents, including heat, the number of individuals dying per unit time is proportional to the number remaining, *i.e.*, the

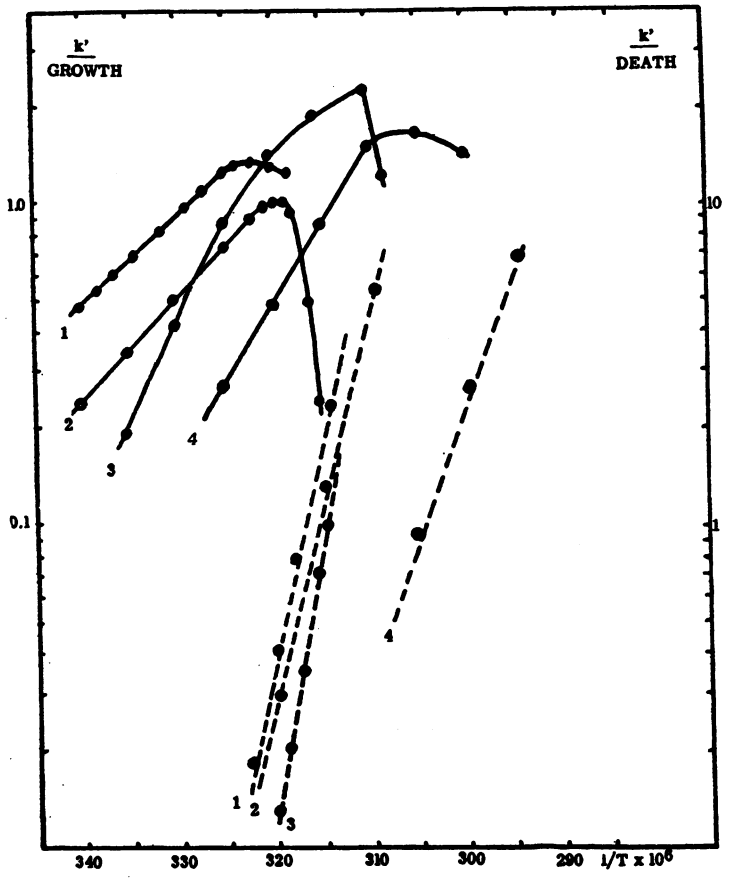

FIG. 5. Effect of temperature on growth and death of several bacteria. Solid lines, measured growth rates; dotted lines, calculated death rates. Curves 1, *Escherichia coli* (Monod); curves 2, *E. coli* (Johnson and Lewin); curves 3, *Lactobacillus delbrueckii*; curves 4, thermophilic *Bacillus circulans*, strain 7-2. The rate constants for growth and death are plotted on a logarithmic scale vs. 1/T.

death curves are exponential, even from the start. Although some workers have reported nonexponential killing curves (39, 176), there appears to be little doubt that death as a logarithmic function of time is the relation commonly observed with bacteria.

The best explanation which has so far been advanced for exponential killing curves is that the organisms are destroyed by the inactivation of one, or at most

a very few, critical catalysts in each cell, rather than by the denaturation of components, such as the respiratory enzymes, which are present in large quantity. Whether the key molecules are the catalysts responsible for the synthesis of enzymes, materials necessary for the maintenance of structural patterns, or enzymes present in a concentration of one or two molecules per cell is a problem for further work. This problem is, however, accessible to study. For example, the effect of temperature on enzyme synthesis could be measured with adaptive enzymes.

Knowledge of the thermophilic microorganisms appears by now to have reached the point where the organisms are recognizable and their relation to other bacteria is reasonably clear, and where the existence of thermophilic variants of well known mesophilic forms makes possible comparative studies on controlled material. Because of these facts, closer physicochemical and chemical study of some factors which permit growth at high temperatures may be envisaged. These admittedly speculative closing remarks are offered in the hope that they may stimulate further research in this direction.

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Note added in proof: Since this manuscript was completed Smith, Gordon, and Clark (155a) have published a revision of their monograph on aerobic sporeforming bacteria, presenting an integrated treatment of thermophils and mesophils, and establishing *Bacillus licheniformis* as a separate species. Also, Campbell and Williams (34a) have reported the nutritional requirements of several thermophilic sporeformers. Some of their strains had nutritional requirements independent of temperature, some were more nutritionally demanding at high temperatures, while others required more growth factors at low temperatures. The isolates varied widely in their vitamin and amino acid requirements.

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